PROLACTIN-INDUCIBLE-PROTEIN (PIP) INFLUENCES HOST IMMUNITY BY REGULATING INTRACELLULAR SIGNALING PATHWAYS IN MACROPHAGES

By

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ABSTRACT

The human prolactin-inducible protein (PIP) or gross cystic disease fluid protein -15 (GCDFP-15) is a 15 kD protein secreted by human breast cancer cells and is abundant in fluids from gross cystic breast disease. Previous results from our laboratory showed that PIP KO mice had significantly lower numbers of $CD4^+$ T cells in their secondary lymphoid organs, and these cells are impaired in their ability to differentiate into Th1 cells *in vitro* and *in vivo* leading to failure to control *Leishmania major* infection. In the present study, we further assessed the role of PIP in adaptive immunity by comparing cytokine production and intracellular signaling events in macrophages from WT and PIP KO mice following IFN-γ and lipopolysaccharide (LPS) stimulation. We show that although the expressions of IFN-γR and TLR4 on macrophages from KO and WT mice were comparable, PIP KO macrophages were significantly impaired in producing proinflammatory cytokines following IFN-γ and LPS stimulation. This was associated with impaired phosphorylation of mitogen-activated protein kinases (MAPKs) and signal transducers of activation of transcription (STATs) proteins in IFN-γ and LPS-stimulated macrophages from PIP KO mice. Interestingly, the expression of suppressors of cytokine signaling (SOCS) 1 and 3 proteins, known to suppress IFN-γ and LPS signaling, was higher in PIP KO macrophages compared to those from WT mice. Collectively, our studies clearly show that deficiency of PIP significantly affects intracellular signaling events leading to proinflammatory cytokine production in macrophages, and further confirm a role for PIP as important immunoregulatory protein involved in host defense.

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ABBREVIATIONS

µg	microgram
µl	microliter
ACK	Ammonium-Chloride-Potassium Lysing Buffer
APC	Antigen presenting cells
BCA	Bicinchoninic acid assay
BMDDC	Bone marrow derived dendritic cells
BMDMs	Bone marrow derived macrophage cells
BSA	Bovine serum albumin
CD1 mice	Cluster of differentiation 1 mice (Outbred mice)
CD11b+	Marker of macrophages
CD11c+	Marker of dendritic cells
CD14	Cluster of differentiation 14
CD4	Cluster of differentiation 1
CD4+T - cell	Helper T- cells
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 ligand
CD8+ T- cells	Cytotoxic T- cells
cDNA	Complementary DNA
CL	Cutaneous leishmaniasis
DAMPs	Danger-associated molecular patterns
DC	Dendritic cells
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Sandwich enzyme-linked immunosorbent assays
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERK1/2	Extracellular signal regulated kinase 1/2
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
GCDFP-15	Gross cystic disease fluid protein - 15
gp-17	Glycoprotein 17
HER2	Human epidermal growth factor receptor
HIV-1	Human immunodeficiency virus -1
hPIP	Human prolactin inducible protein
HRP	Horseradish peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IFN-g	Interferon gamma
IFN-gR	Interferon gamma receptor
IKB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL-1	Interleukin 1

IL-12	Interleukin -12
IL-12R	Interleukin -12 Receptor
IL-13	Interleukin -13
IL-4	Interleukin -4
IL-6	Interleukin -6
IL-6R	Interleukin -6 Receptor
iNOS	Inducible nitric oxide synthase
IRAK1	Interleukin-1 receptor-associated kinase-1
IRAK4	Interleukin-1 receptor-associated kinase-4
IRF3	Interferon regulatory factor-3
JAK1	Janus kinase1
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinases
KHCO3	Potassium bicarbonate
LCCM	L929 conditioned medium
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinasekinase
MAPKs	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony stimulating factor
MD-2	Lymphocyte antigen 96
Mg	Milligram
MHC	Major Histocompatibility complex
MHC-II	Major Histocompatibility complex -II
Ml	Milliliter
mPIP	Mouse prolactin inducible protein
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa- B
Ng	Nanogram
NH4CL	Ammonium chloride
NK	Natural killer cells
NKSF	Natural killer cell stimulatory factor
NLRs	NOD-like receptors
nNOS	Neuronal Nitric oxide synthase
NO	Nitric oxide
OVA	Ovalbumin
PAGE	Polyacrylamide gel Electrophoresis
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pg	Picogram
PIP	Prolactin inducible protein

PIP KO	Prolactin inducible protein knockout mice
PMSF	Phenylmethanesulfonylfluoride
PR	Prolactin receptor
PRRs	Pattern recognition receptors
PVDF	Polyvinylidenediflouride
RIG	Retinoic acid-inducible gene
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMGP	Submandibular gland protein
SOCS1	Suppressor of cytokine signaling 1
SOCS3	Suppressor of cytokine signaling 3
STAT1	Signal transducer and activation of transcription 1
STAT3	Signal transducer and activation of transcription 1
SVA 1	Seminal vesicle autoantigen 1
SVA 2	Seminal vesicle autoantigen 2
T47D	Breast cancer cell line
TAB1	TAK1-binding protein 1
TAB2	TAK1-binding protein 2
TAB3	TAK1-binding protein 3
TBXT	T-cell differentiation factor
TCDF	T cell differentiation factor
TCR	T cell receptors
Th1	T- helper 1 subset
TICAM -2	TIR-containing adapter molecule-2
TICAM-1	TIR-containing adapter molecule-1
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adapter protein
TIRP	TIR-containing protein
TLR4	Toll like receptors 4
TLRs	Toll like receptors
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TNF-α	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor (TNF) receptor associated factor-6
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter inducing interferon-b
V-CAM1	Vascular cell adhesion molecule 1
WT –	WT mice
ZR-75	Breast cancer cell line

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

1. BACKGROUND

1. THE HUMAN PROLACTIN INDUCIBLE PROTEIN (PIP)

The human prolactin inducible protein (PIP) is an acidic protein reported by Haagensen *et al*. (Haagensen *et al*., 1986). PIP was independently identified as a glycoprotein produced by the human breast cancer cell lines (T47D) in response to prolactin (Shiu and Iwasiow, 1985; Murphy *et al.*, 1987). PIP is analogous to gross cystic disease fluid protein 15 (GCDFP-15) which is a highly abundant protein present in gross cystic disease fluid of breast (Haagensen *et al*., 1979.; Haagensen *et al.,* 1980). PIP / GCDFP -15 was subsequently shown to be highly expressed in apocrine metaplasia of the breast but rarely expressed from normal ductal or lobular epithelium (Mazoujian *et al.*, 1983). Thus, PIP / GCDFP-15 have been routinely used as an immunohistochemistry marker for evaluating potential mammary origin of metastatic carcinoma of unknown primary site. This protein was also later identified by several other groups as human seminal fluid gp 17, a 17-kDa actin binding protein present in human seminal fluid (Autiero *et al*., 1991), and as an abundant protein found in human submandibular/and sublingual saliva and it is referred to as the extra parotid glycoprotein (Rathman *et al*., 1989; Schenkels *et al*., 1993).

1.1. PIP homologues

Gross sequence analysis of human PIP with that of other mammalian sequences available in gene bank database reveals a number of homologous sequences as in other species. Myal *et al* first identified PIP sequences in human and non – human species (Monkey, dog, cow, rabbit, chicken, yeast) using Southern blot analysis (Myal *et al*., 2000). It was later reported that human PIP shows sequence identity of 97% with chimpanzee (Kitano *et al.,* 2006), 94% with gorilla (Kitano

et al., 2006) 93% with orangutan (Kitano *et al.,* 2006), 90% with gibbon (Kitano *et al.,* 2006), 89% syndactylus (Kitano *et al*., 2006), 71%with Japanese monkey (Osawa *et al.,* 2004), 61%with guinea pig (Osawa *et al*., 2004) , 55% with rabbit (Osawa *et al*., 2004) , 52% with bovine (Osawa *et al*., 2004), 51% with mouse (Windass *et al*., 1984) and 38% with rat (Mirels *et al.,* 1998).

1.2. Expression and regulation of PIP

Most organs of the body that are involved in secretion of bodily fluids are known to express PIP gene. The highest expression of PIP gene has been observed in the salivary gland, followed by lacrimal gland, prostate gland, muscle, trachea, mammary gland, lung and other organs (Hassan *et al*., 2008). Pathologically, PIP has been reported to be abundantly expressed in breast cyst fluid, (Haagensen *et al*., 1979; Haagensen, 1981) and in human breast carcinomas. PIP is specifically found in secretion from apocrine glands of the axilla, saliva, perineum, moll's gland of the eyelids and the ceruminous glands of the ear (Mazoujian *et al.,* 1983; Haagensen *et al*., 1981). As well as saliva, tears, sweat, amniotic fluid, breast milk, blood, and fluid from the seminal vesicle and bronchial submucosal glands contain human PIP as their main component relative to other protein (Murphy *et al.,* 1987).

In both normal and diseased state of breast, the expression of the PIP gene is shown to be regulated by many hormones and cytokines. For example, PIP is up-regulated by androgens, prolactin, glucocorticoids, and progesterone (Shiu and Iwasiow, 1985) and bit has been shown to be influenced by the cytokines IL-4/IL-13 in breast tumors (Blais *et al*., 1996).

1.3. Proposed function of PIP

To date the role of PIP is not clearly elucidated; however several studies suggest that its role is multifunctional. PIP is suggested to play a potential role in immunomodulation, inhibits proliferation of bacteria, infertility, prostate and breast tumor progression and formation of enamel pellicle (Rathma *et al.,* 1989; Rathman *et al*., 1990). More recently, it has been suggested that PIP may play a role in innate and adaptive immunity (Umadat *et al.,* 2013).

1.4. Crystalline structure of PIP

The recent determination of the crystal structure of PIP (Hassan et al., 2009) has provided important insights into a possible role in immunity. The crystalline structure has revealed an immunoglobulin fold composed of seven antiparallel beta-strands and seven loops (Hassan et al., 2009). The identification of the beta folds is consistent with the hypothetical model previously proposed by Caputo *et al.* (Caputo *et al.,* 2000) who used a homology modeling approach to elucidate a three dimensional model of the PIP protein. The beta fold in PIP is thought to play a major role in the interaction and binding to numerous immunoregulatory molecules such as the CD4 molecule on T cells, immunoglobulin G (IgG), actins, and zinc α2-glycoprotein (ZAG), as well as non-immunomodulatory molecules, fibronectin and enamel pellicle. (Anderson *et al.,* 2006; Hassan *et al.,* 2009). This suggests that PIP might be an immune molecule that plays a role in maintaining host immunity.

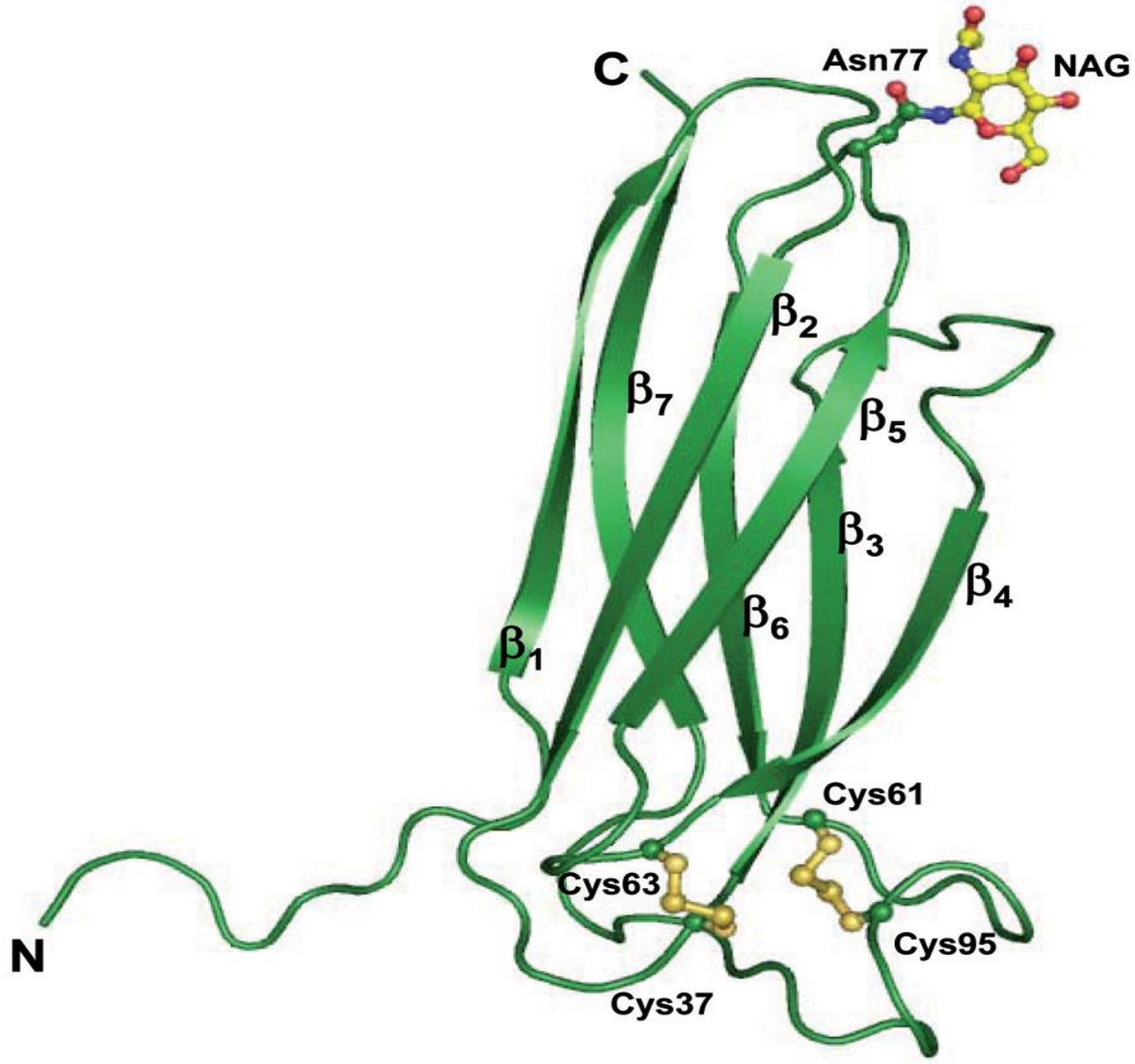


Figure 1. **Three-dimensional structural fold of PIP represents seven b-stranded structures**.
A single potential glycosylation site (Asn77) which is accommodated by N-acetyl glucosamine (NAG) shown in ball and stick (yellow).Two disulfide bonds formed by four cysteine residues are indicated in ball and stick (dark yellow).
Modified from Hassan et al., 2008

2. PIP AS AN IMMUNOREGULATORY MOLECULE

2.1. Innate Immunity

The innate immune system, otherwise known as the natural or first line of host defense, is present at birth and relies on receptors commonly found in body fluids, tissue cells or mobile hematopoietic cells to detect microbial pathogens (Paul, 2008). The innate defense is quick but lacks specificity. Among the cells that bear innate immune receptors are macrophages, dendritic cells, mast cells, neutrophils, eosinophils and natural killer (NK) cells (Janeway and Medzhitov, 2002). The innate immune system protects the host from pathogen entry by providing a physical barrier and producing molecules such as mucins and other microbicidal products that help prevent the entry, attachment, and growth of harmful agents (Paul, 2008).Many salivary components have the ability to interact with bacterium which frequently inhabit the oral cavity and deter their expansion. This property makes saliva an integral part of the innate immune defense system. Molecules in saliva that contribute to this interaction include alpha-amylase, immunoglobulin A, mucins, proline-rich glycoprotein, histatins, and lysozyme (Schenkels *et al*., 1997). PIP, which is found in saliva, can be listed among these molecules, and as such, is thought to play a role in the first line of immune defense against invading pathogens (Shiu *et al*., 1987).

2.2. PIP in innate immunity

PIP is found strategically located at several ports of pathogen entry and its predominance in mucosal type tissues, as well as its presence in saliva, tears, submucosal glands of the bronchi and apocrine glands of the skin, suggests that PIP may play an important role in mucosal

immunity and in the first line of innate immune defense (Haagensen and Mazoujian, 1986). Previous studies has demonstrated that PIP, which is secreted by the salivary glands has the ability to bind *in vitro* to the surface of bacterial strains *(Gemella*, *Staphlococcus* and *Streptococcus)* which are colonizing the oral cavity, ear canal and skin resulting in the inhibition of bacterial growth (Schenkels *et al*., 1997).

2.3. Adaptive immunity

Adaptive immunity otherwise known as the acquired or specific immunity relies on the expansion of lymphocytes that express highly diverse clonally restricted antigen-specific receptors arising from rearrangements of gene segments encoded in specialized genetic loci (Paul, W.E. 2008). The function of the adaptive immune response is to destroy invading pathogens and any toxins they produce. This system targets specifically foreign entities to prevent destruction of host defenses (Alberts *et al*., 2002). The key mediators in the adaptive response are white blood cells known as lymphocytes that produce two broad classes of responses (Alberts *et al*., 2002). The cell-mediated response is dependent on T lymphocytes (Alberts *et al*., 2002). T cell activation for subsequent effector functions is regulated by their interaction with dendritic cells, which are specialized antigen-presenting cells that are critically important for T cell recognition of antigens. Dendritic cells express toll-like receptors (TLR), which specifically recognize discrete pathogen associated molecular patterns. Ligation of TLRs on dendritic cells leads to their activation, the production of inflammatory cytokines such as IL-6, IL-12, tumor necrosis factor (TNF) and expression of co-stimulatory molecules (Warger *et al*., 2006).

2.4. PIP in adaptive immunity

The binding and interaction of PIP to several components of the adaptive immune system has led to the speculation that it may be an important component of the adaptive immune system. PIP interacts with the CD4 molecule on T cells. CD4 molecule acts as a signaling molecule during T cell activation and is a co-receptor for the interaction of T cell receptor (TCR) with the major histocompatibility complex class-II (MHC-II) molecule on antigen-presenting cells (Zhou and Konig, 2003).CD4 is also the primary viral receptor for Human Immunodeficiency Virus-1 (HIV-1) (Klatzmann *et al*., 1984; Maddon *et al.,* 1986) that allows viral entry into T-helper lymphocytes. The interaction of CD4 with the HIV-1 envelope glycoprotein gp120 mediates attachment and penetration of the virus into the cell (McDougal et al., 1986). Autiero *et al.* (Autiero *et al*., 1997) showed that the interaction of PIP with the first domain of CD4 induces a conformational change that disrupts the binding of HIV retrovirus to CD4+ cells and the post-binding events that follow. Also, it is well established that HIV infection induces immunosuppression by causing a loss of CD4+ T cells and that the interaction of PIP with CD4 on the T cell, blocks the forced apoptotic pathway that would normally occur (Gaubin *et al.,* 1999). Thus, PIP has the ability to inhibit this process, helping to sustain the adaptive immune response of the host that would otherwise be disturbed by the sequential CD4/TCR triggering during a viral infection. PIP has also been reported to bind to the D1-D2 region of CD4 with high affinity in human seminal plasma (Autiero *et al*., 1991; Autiero *et al*., 1997). Human seminal plasma is known to have a wide array of effects on immune function. It is therefore possible that PIP interaction with CD4 may contribute to the immunosuppressive properties of human seminal plasma. It has been demonstrated that both IL-4 and IL-13 could act in breast cancer cells to regulate proliferation and protein expression (Blais *et al.,* 1996). Furthermore, NK cells isolated

directly from the tumor site were shown to secrete an abundance of IFN-γ and IL-4 when compared to the cells collected from the patient's blood (Lorenzen *et al.*, 1991). It has also been demonstrated that exposure of the breast cancer cell line, ZR-75, to IL-4 and IL-13, up-regulated PIP mRNA levels 5.5 and 6.0 fold, respectively (Blais *et al*., 1996). Due to the observation that PIP binds to CD4 domains that interact with MHC-II molecules (that are involved in antigen recognition) (Autiero *et al*., 1995). It has been speculated that the increase in PIP expression in response to IL-4 and IL-13 levels may modulate the activity of infiltrating CD4+ T cells, and thus contribute to the innate and adaptive immune response (Blais *et al*., 1996).

3. THE MAMMARY GLAND AS AN APOCRINE GLAND

Apocrine gland is a type of gland that is found in the skin, breast, eyelid, and ear. An apocrine gland consists of two parts:

1. A coiled secretory structure situated in the lower part of the dermis or in the subcutaneous fat.
2. A straight duct that enters an infundibulum.

Lumen of an apocrine gland is lined by a layer of cuboidal or columnar cells while secretory cells of an apocrine gland are surrounded by a single layer of myoepithelial cells. Apocrine glands secrete its products by decapitation, thus a mammary gland can be identified as an apocrine gland because the manner of secretion is by pinching off or decapitation of apical cytoplasm. Also, the duct of a mammary gland, named the lactiferous duct, looks just like any other apocrine duct.

Apocrine glands are small and nonfunctional until puberty, at which time they enlarge and begin to secrete their product. Apocrine glands in the breast secrete fat droplets into breast milk.

3.1. Human PIP/ GCDFP- 15 in the context of breast cancer:

Worldwide, breast cancer is known as the most common invasive cancer in women (www. who.int). It has also been recorded that breast cancer is responsible for 22.9% of invasive cancers in women and 16% of other female cancers (world cancer report, 2008).

Breast cancer is a heterogeneous disease and numerous molecular subtypes of breast cancer have been identified using biological markers (Perou CM, *2000*). These subtypes are classified based on the presence and absence of estrogen(ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) (Reis-Filho and Pusztai, 2011). The first five subtypes identified were:

1. Luminal A- subtype (ER+ / PR+ / HER2-)
2. Luminal B- subtype (ER+/PR- HER2 +)
3. HER2 Positive (ER- / PR-)
4. Basal like subtype
5. Normal like subtype

The luminal A- subtype of breast cancer which is ER+ and the luminal androgen receptor positive which is associated with breast cancer has been shown to express PIP protein (Farmer *et al.,* 2005; Baniwal *et al*., 2013). Recently, ten subtypes have been identified as well as a Claudin – low subtype. Many of these subtypes are poorly characterized. Another group of tumors are the triple negative breast cancer (TNBC), which has recently been described to consists of at least six molecular subtypes (Lehmann *et al.,* 2011).

Two basal-like (BL1 and BL2)

Two mesenchymal namely Mesenchymal (M) and mesenchymal stem- like (MSL)

A luminal androgen receptor (LAR) positive

An immunomodulatory subtype (IM).

The Immunomodulatory subtype of TNBC is associated with upregulated expression of genes involved in Th1/Th2 immune responses, antigen processing, immune effector functions and inflammatory pathways (Bertucci *et al*., 2006; Allard *et al*., 2014).

4. MOUSE PIP (mPIP)

mPIP also known as mouse submaxillary gland protein (mSMGP) is a 14 kDa protein and is an established murine homologue of hPIP (Windass *et al*., 1984; Myal *et al.,* 1994). mPIP exhibits 51% identity at the amino acid level with hPIP (Myal *et al*., 1991). In terms of tissue specific expression, mPIP displays a high level of similarity to its human counterpart. Like the human PIP, mPIP expression was found to be strategically associated with tissues located at several ports of pathogen entry. Importantly, the activities of both the human and mouse PIP protein bear much similarity with regards to function. The mRNA of mPIP is highly expressed in both salivary and lacrimal glands. Thus, mPIP is found abundantly in the secretions of these glands, namely saliva and tears (Myal *et al.,* 1994; Blanchard *et al.*, 2004).

mPIP gene is located on chromosome 6q34 and it share a common structure with four other genes such as seminal vesicle auto antigen (SVA) and SVAL (SVA – like) 1-3 (Osawa *et al*., 2004). SVA is a 19-kDa glycoprotein found in the seminal plasma where it plays a role of preventing spermatozoa mobility by its interaction with cell membrane phospholipids (Huang *et al*., 1999).In addition, they also share many amino acids, though SVA, SVAL1 and SVAL2

protein show low amino acid identities of about (25–30%). The sharing of common structure and amino acids is specific only in mouse genome, however PIP from other mammals lack family members and does not share their structure or amino acids with any other amino acids (Osawa *et al.,* 2004). In the mouse, the SVA proteins are expressed in different tissue besides lachrymal and salivary gland. The mPIP expression, as shown by RT-PCR, originates from lachrymal and major salivary glands, however SVA and SVAL2 are expressed in seminal vesicles and lactating mammary gland (Osawa *et al.,* 2004).

Consistent with hPIP, the main function of mPIP is yet to be defined. mPIP can specifically bind to several bacterial strains in both human and mouse, including some within the genus *Streptococcus*, which are known to inhabit the oral cavity (Lee *et al*., 2002). The binding of mPIP to the bacteria promotes bacterial aggregation and in so doing, possibly inhibiting further proliferation and colonization of the oral cavity (Lee *et al.*, 2002). Such studies provides further evidence to the hypothesis that mPIP has an antibacterial activities related to host defense. Additionally, expression of the mPIP gene from the mouse submaxillary gland at the early stage of embryonic development suggests that mPIP may contribute to the development of submaxillary gland (Lee *et al*., 2003).

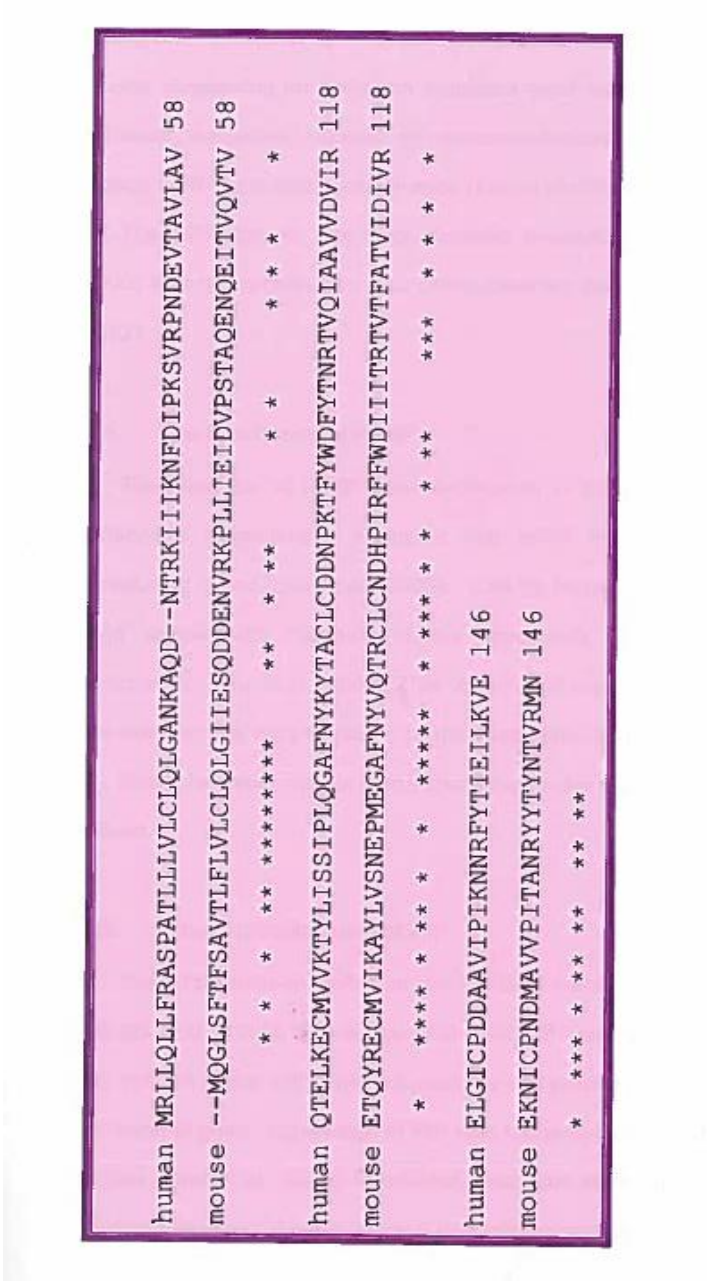


Fig 2. Amino-acid sequence alignment of mouse and human PIP.

There is 46.6% identity at the amino- acid level between mouse and human PIP

4.1. Generation of PIP KO mouse model

Mouse models are commonly used for the study of human biology and disease compared to other model organisms such as worms, flies and zebrafish as they can easily be genetically modified and some of the diseases is more closely resemble what we find in human. The similarity of mPIP with hPIP in terms of site of expression (Windass *et al.* 1984; Myal *et al*., 1998), location and function led to the generation of a PIP knock out (KO) mouse model to study the function of hPIP *in vivo*.

The PIP KO mice were generated using gene targeted disruption strategies. The hPIP gene has 4 exons (Myal et al., 1991). Generation of PIP KO mice was done by removing exon 2 (one of the four exons) from the gene targeting construct, followed by replacement using phosphoglycerate kinase promoter and neomycin resistant (PGK-neo) gene cassette. PGK – DTA (Diphtheria toxin A- fragment gene) cassette play a role as a negative selection maker and the differentiation of WT type and mutant allele was done using PCR (Blanchard *et al*., 2009).

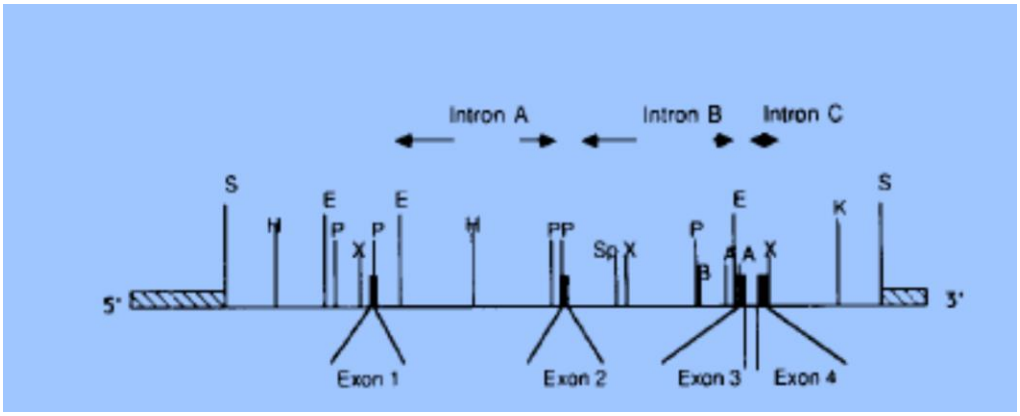


Fig 3. The 7kb long of PIP gene showing 4 exons and three introns.
Restriction enzymes are as follows: S = SaII; H = HindIII; E = EcoRI; P=PstI; Sp=Sphl; X = Xbal; K = KpnI; A = Accl; B = BamHI

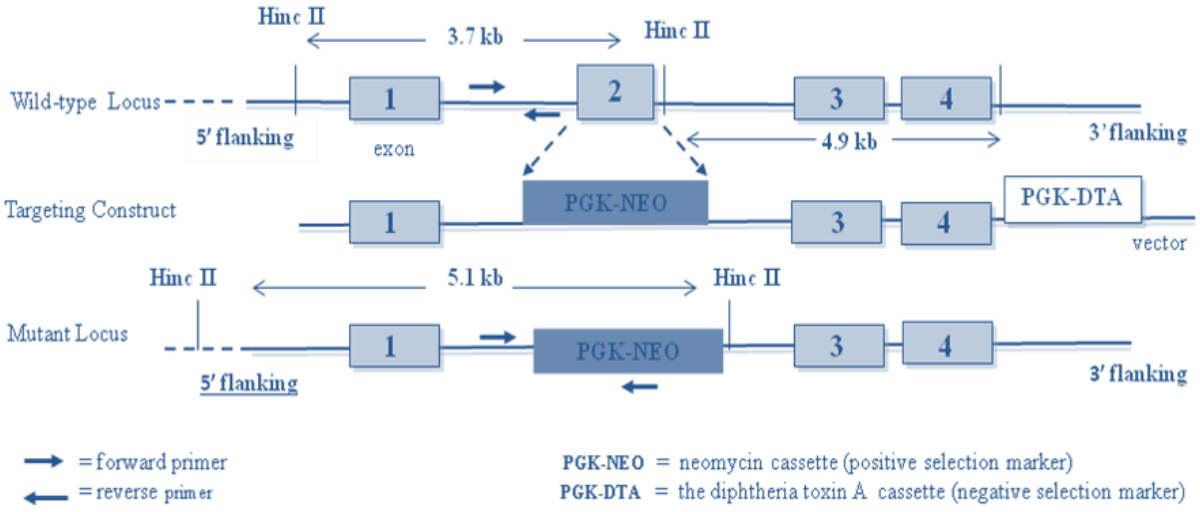


Fig 4. Generation and PCR detection strategy of the mPIP knockout mouse.

4.2. Phenotypic features of PIP KO mouse model

Gross appearance of PIP KO mice showed no overt phenotypic changes. They are active and appear to grow / develop normally without gross lesion. In addition, the PIP KO mice are fertile and have similar life expectancy compared with the wild type (WT) mice. However, histological analyses and findings revealed changes in PIP KO mice that were absent in WT mice. Some of these findings include: enlarged submaxillary gland lymph node and thymic medulla with many germinal centers, lymphocytic aggregation in the prostate gland, heart with signs of arteritis and periarteritis, Ulcerative colitis and lymphocytic infiltration of the gut associated lymphoid tissue of the large intestine (Blanchard *et al*., 2009).

In addition, recent findings from PIP KO mice have shown that naïve CD4+ T cells from PIP deficient mice are impaired in their proliferation and differentiation into IFN-γ producing cells following *in vitro* polarization (Li *et al.*, 2015). Also production of IFN-γ by lymphoid cells from PIP deficient mice following *in vivo* immunization with ovalbumin (OVA) was significantly impaired compared to their WT counterpart cells. Furthermore, the production of nitric oxide and proinflammatory cytokines (IL-6, IL-1, IL-12, TNF) by IFN-γ activated dendritic cells from PIP deficient mice is impaired compared to those from their WT counterpart mice, thus resulting to their susceptibility to *Leishmania major* (Li *et al*., 2015).

1. THE IMMUNE SYSTEM

2. INNATE IMMUNE CELLS

The innate immune cells arise from the multipotent hematopoietic stem cells present in the bone marrow. They primarily function within the immune system by identifying and eliminating pathogens that might cause infection. The major innate immune cells include natural killer (NK) cells, eosinophils, basophils, mast cells and phagocytes.

Phagocyte is a general name for granulocytes (monocytes, macrophages and dendritic cells) and these cells play a critical role in resistance against invading organism by mediating phagocytosis and degradation of microbes. Phagocytes express surface receptors for pathogen recognition and utilize these receptors to initiate intracellular signaling events that result in antimicrobial function (Silverstein *et al.,* 1989).

6.1. Dendritic cell (DC)

Dendritic cells are phagocytes present in tissues that are in contact with the external environment such as the skins, inner linings of the mucosal surfaces and in specialized compartments within the lymphoid organs. They are named for their resemblance to dendrites seen in neurons due to their numerous projections, which is a marker of their activation. DCs are rare, not abundant and are not uniformly distributed throughout the tissues as macrophages. Like all phagocytes, DCs originate from hematopoietic stem cells in the bone marrow and are referred to as either immature or mature base on their function and phenotypic characteristics. An important characteristic of an immature DC is their ability to efficiently capture and phagocytose antigens (Mellman and Steinman, 2001). Following antigen capture, immature DCs undergo

transformational changes (referred to as maturation) and become mature DCs, which is associated with changes in their phenotypic and functional features. Mature DCs are incapable of antigen uptake. However, they are excellent in antigen (Ag) presentation, and thus have a higher capacity for activating naïve T cells to initiate adaptive immune responses (Banchereau and Steinman, 1998; Banchereau *et al.,* 2000). This is because maturation of DCs results in increased expression of major histocompatibility complex (MHC) molecules on their surface membrane (Inaba *et al*., 2000; Turley *et al.,* 2000),upregulation of expression of co-stimulatory molecules (such as CD40, CD80, and CD86) and increased production of pro-inflammatory cytokines (Banchereau and Steinman, 1998).

The pro-inflammatory cytokines released by the DC are responsible for the polarization of the T cell response into different forms of cell-mediated immunity (Cools *et al*., 2007; Joffre *et al*., 2009). These pro-inflammatory cytokines include interleukin (IL)-12, IL-6, TNF, IL-10, etc. In particular, the production of IL-12 by DCs is critically important for the differentiation of naïve T cells into Th1 cells that produce IFN-γ, a critical cytokine for the development of resistance to *Leishmania major* in mice. In contrast, the failure of DCs to produce IL-12 results in Th2 differentiation and susceptibility to the infection.

6.2. Macrophages

Macrophages are phagocytic cells (monocytes) that able to move outside of the vascular system by moving across the walls of capillaries and enter into the tissues to seek out and destroy pathogens. They are the most efficient phagocytes, and can phagocytose substantial numbers of bacteria or other cells or microbes. Like other phagocytes, macrophages recognize pathogens

through several innate (pattern recognition) receptors. This recognition usually results in phagocytosis and subsequent activation. This activation results in the generation of respiratory burst, which results in the release of several reactive oxygen and nitrogen intermediates. This activation process can be facilitated by several inflammatory cytokines such as IFN-γ and tumor necrosis factor (TNF).

6.3. Macrophage activation

Activation is a pre-requisite for macrophages to mediate effector functions such as intracellular pathogen destruction or production of cytokines. Recent studies has shown that activated macrophages are heterogeneous based on the nature of activating stimuli and this results in different immunological outcomes. There are two main types of activated macrophages; classical and alternative.

Macrophages become classically activated following exposure to two cytokine signals. The first cytokine is IFN-γ, which is obligatory and it is known to prime macrophages for activation but rarely activates macrophages alone (Nathan, 1991). The second signal is TNF, which could be obtained exogenously or endogenously from the macrophage itself by ligation of toll like receptor (TLR) and lipopolysaccharide (LPS). Thus, classically activated macrophages are developed from the priming effect of IFN-γ, followed by exposure to a microbe or microbial product such as LPS. Studies in mice showed that classically activated macrophages produce nitric oxide (NO) (Hibbs, 2002). However, a recent report showed that macrophages that have been primed with IFN-γ alone do not make NO in an LPS free system. Classically activated macrophages are known to possess remarkable abilities to kill and degrade intracellular

microorganisms. Killing of intracellular microbe is mainly accomplished by enhanced production of toxic oxygen species and induction of the inducible NO synthase (iNOS) gene to produce NO. In addition, studies have shown that microbial killing could also be due to restriction of nutrients such as iron (Gruenheid and Gros, 2000) and tryptophan (Carlin *et al*., 1989) from the phagosome, which limit the growth of the intracellular microbe. Although this second method of killing is under appreciated and is not fully documented, compared to microbial killing by NO, it is still an important aspect of microbial killing that is used by the classically activated macrophages. Interestingly, it has been shown that products of classically activated macrophage are capable of causing extensive tissue damage to the host if not regulated. Thus, classically activated macrophage has been associated with immunopathologies such as type 1 autoimmune diseases (Flavell, 2002). Therefore, the activities of classically activated macrophages must be tightly regulated in order to prevent immunopathology.

Alternatively activated macrophages derived their name from the fact this is an alternative way of activating macrophage aside from the classical method (i.e. due to non-exposure to classical activating cytokines such as IFN-γ and LPS). The exposure of macrophages to interleukin 4 (IL-4) inhibits macrophages from making nitrogen radicals (NO) (Modolell *et al*., 1995), thus making the cell to be relatively poor at killing intracellular microbes. These studies showed that alternatively activated macrophage fail to make NO by virtue of their induction of arginase (Rutschman *et al*., 2001), thereby compromising its ability to kill intracellular microbes leading to immunosuppression. The major cytokines produced by alternatively activated macrophages are IL-10 and IL-1 receptor antagonist, key cytokines that have potent immunoregulatory properties.

Recent studies have shown that alternatively activated macrophages also produce numerous molecules that aid the synthesis of extracellular matrix (ECM) (Gratchev *et al*., 2001), strongly suggesting that the main role of alternatively activated macrophage is in wound healing, angiogenesis and tissue repair. The induction of arginase in these cells does not only suppress INOS synthase and NO production, it may lead production of polyamine and proline thereby enhancing cell growth, collagen formation, and tissue repair (Hesse *et al*., 2001). Thus, alternatively activated macrophage induced by IL-4-treated macrophages (Raes *et al*.,2002) functions as a regulatory and recovery cell than the effector killing functions that are associated with classically activated macrophages.

6.4. Role of IFN-γ as macrophage activating factor

IFN-γ is known to be produced by cells involved in immunity and inflammation such as CD4+ Th1 subsets, CD8+ (cytotoxic) T cells, natural killer (NK) cells, B cells, professional antigen-presenting cells (APCs) and NKT cells (Schroder *et al*., 2004). IFN-γ released by NK cells and antigen presenting cells (APCs) play a crucial role in the early stage of host defense against invading microbes (innate immune response); while T lymphocytes produce larger quantity of IFN-γ during adaptive immune response. Following exposure of macrophages to pathogen, chemokines released by macrophages attracts NK cells to the inflammatory site, as well as IL-12 that enhances the production of IFN-γ by these cells. Thus IL-12 and IL-18 produced by the activated immune cell such as NK cells, macrophages, and T- cells work synergistically to stimulate production and release of IFN-γ. IFN-γ activates macrophage by binding to IFN-γ receptor (IFN-γR) expressed on the surface of macrophages and other responsive immune cells.

The IFN-γR is a heterodimer that consist of IFN-γR1 and IFN-γR2 (ref). The intracellular domain of IFN-γR1 is associated with the inactive janus kinase (Jak) 1 and 2 proteins. Following dimerization of IFN-γR1 and IFN-γR2 (which is initiated by IFN-γ binding to IFN-γR1), Jak 1 and Jak 2 transactivate each other leading to their phosphorylation. Phosphorylated Jak1 and Jak2 form a docking site for Stat1 proteins. The docking results in phosphorylation of the Stat1 proteins, their translocation into the nucleus and subsequent binding to the promoter region of interferon gamma inducible genes.

3. INNATE IMMUNE RECEPTORS

Recognition of pathogens by the innate immune system is mediated by a number of germline-encoded receptors, which detect molecular patterns present on / in the invading pathogens, collectively known as pathogen-associated molecular patterns (PAMPs). The receptors that detect these structures are called pattern recognition receptors (PRRs) (Akira *et al*., 2006). PRRs are expressed on the cell surfaces of innate immune cells. PRRs are broadly divided into two:

1. Transmembrane proteins such as Toll-like receptors (TLRs) and C-type receptors (CLR)
2. Cytoplamic proteins such as Retinoic acid-inducible gene I (RIG)-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

The TLR family is the most characterized of all PRRs. The detection of PAMPs by PRRs induces an inflammatory response, thus resulting in rapid innate immune response. In addition, signal from the activated receptors play a key role in the induction of and expression of costimulatory molecules on professional APCs, thus resulting in the generation of an effective immune response against pathogens.

7.1. Toll-like receptors (TLRs)

TLRs are members of the type-1 transmembrane receptor family that are evolutionarily conserved proteins among vertebrates and invertebrates (Werlin *et al;* 2003). TLRs are homologues of the drosophila toll protein, which has been shown to be important for defense against microbial infection (Medzhitov *et al*., 1997). The gene Toll, known for its role as a dorsoventral regulator of drosophila development, was also involved in the innate immune response of the fly to fungi and gram-positive bacteria (Lemaitre *et al.,* 1996; Lemaitre *et al.,* 1997). TLRs recognize highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs), which are highly expressed by microbial pathogens, or danger-associated molecular patterns (DAMP).They are transmembrane receptors that has both the extracellular / cell surface domain that contain multiple leucine-rich repeats (LRR) and the cytoplasmic tail that contains a highly conserved region called the Toll/IL-1 receptor (TIR) domain that is crucial for signal transduction (Janssens *et al*., 2003; Kanzler *et al*., 2007). TLRs are highly expressed in immune responsive tissue such macrophages, dendritic cells, spleen cells as well as cells of the tissues that are exposed to the external environment such as lungs and the gastrointestinal tract. The level of expression varies among the different cell types. Previous work has identified some members of TLRs family (TLR4 and TLR2) as the cell-surface receptor for LPS (the outer membrane of gram-negative bacteria), which phosphorylates NF-kB; the activated NF-kB translocate to the nucleus and induces the transcription of proinflammatory genes produced by macrophages (See Section 8 below).

7.2. Toll like-receptor 4 (TLR4)

Structurally, TLR4 has both extracellular domain containing leucine-rich repeats (LRRs) that aid in ligand binding recognition and the intracellular TIR domain involved in cell signaling. TLR4 gene was first identified in the LPS chromosomal region in some mouse strain and its activation mainly leads to the synthesis of pro-inflammatory cytokines and chemokines. In other word, it specifically recognizes LPS. Furthermore, in addition to the recognition of LPS, TLR4 from both mice and human recognizes several other exogenous (Ohashi *et al.,* 2000; Haynes *et al*., 2001) and endogenous (Ohashi *et al*., 2000; Vabulas *et al*., 2001; Figueiredo *et al.,* 2007) molecular components of pathogens. These observations showed that those molecules produced in abnormal conditions such as in situation of tissue damage has the capability to trigger TLR4-dependent pathways (Werling *et al*., 2003).

At the cell surface membrane, TLR4 forms a complex with multiple other proteins necessary for ligand (LPS) recognition (Janssens *et al*., 2003, Werling *et al*., 2003; Miller *et al.,* 2005). In the serum, LPS initially binds to LPS binding protein (LBP) (Schumann *et al*., 1990). LPB catalysis the transfer of LPS to CD14 receptor. CD14 receptor is a membrane bound pattern recognition and the main receptor for LPS (Wight *et al*., 1990). However, CD14 lacks intracellular domain and thus is incapable of transducing cytoplasmic signals (Lee *et al.,* 1993). To compensate for the limitation of intracellular signal transduction, CD14 interacts with TLR4 to recognize LPS. The complex, LPS-CD14-MD -2 proteins associate with the extracellular domain of TLR4 to initiates intracellular signaling events that lead to production of inflammatory cytokines (Park *et al*., 2009). MD-2 also known as Lymphocyte antigen 96 is a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.

The binding of LPS/MD-2/CD14 complex to TLR4 leads to TLR4 dimerization and conformational changes in the TIR domain interface. This enhances the recruitment of adaptor proteins that binds to the intracellular TIR-domain (Nunez *et al*., 2007). TLR4 activation triggers two intracellular signaling pathways: myeloid differentiation factor 88 (MyD88)-dependent and-independent pathways. The specificity these pathways depends on the type of adaptor molecules recruited to the TIR domain (O'Neill *et al*., 2007). These adaptor molecules include MyD88 TIR domain-containing adapter protein (TIRAP), MyD88-adapter-like(Mal) protein TIR-domain-containing adapter inducing interferon (TRIF), TIR-domain-containing adapter molecule-1(TICAM-1), and TRIF-related adapter molecule(TRAM)/TIR-containing protein (TIRP)/TIR-containing adapter molecule-2 (TICAM-2). All these four adapter protein are necessary for TLR4 mediated immune response.

The TIRAP–MyD88 dependent pathway plays a key role in regulating early NF-kB activation and pro – inflammatory cytokine production such as IL-12. In contrast, the TRIF–TRAM dependent pathway activates the interferon regulatory factor-3 (IRF3) transcription factor that results in the subsequent up-regulation of genes encoding interferons (IFNs) and co-stimulatory molecules. In addition, the TRIF-dependent pathway also activates the synthesis and release of TNF-α. Secreted TNF- α subsequently binds to its receptors resulting in NF-kB activation. This indicates that the TRIF–TRAM pathway is associated with late phase NF-kB activation via IRF3 and TNF-α secretion. The majority of LPS response in macrophages is regulated by MyD88-dependent signaling pathway (Akira *et al*., 2004).

Following MyD88 activation, it recruits IL-1 receptor-associated kinase-4 (IRAK-4) via the death domains to TLR4. The binding of recruited IRAK4 to its receptor complex leads to phosphorylation of IRAK1, thus inducing the kinase activity of IRAK1 (Gan and Li, 2006). This

auto phosphorylation of IRAK1 results in its dissociation from MyD88 (Wesche *et al*., 1997), and subsequent binding of IRAK1 to tumor necrosis factor (TNF) receptor associated factor-6 (TRAF6) (Cao *et al*., 1996) via three major conserved binding domains (Ye *et al*., 2002). TRAF6 play a role in mediating the downstream signaling event of the TNF receptor superfamily (Chung *et al*., 2002, Xu *et al.,* 2004). IRAK1 and TRAF6 complex dissociates from the cytoplasmic domain of TLR4 receptor and subsequently form a complex with transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) and three adapter molecules which are TAK1-binding protein 1, 2 and 3 (TAB1, TAB2 and TAB3). In addition, with TRAF6 activation leads to phosphorylation of MKK and IKK complex resulting in the subsequent activation of MAP kinases (JNK, p38 MAPK) and NF-κB (Fig.5).

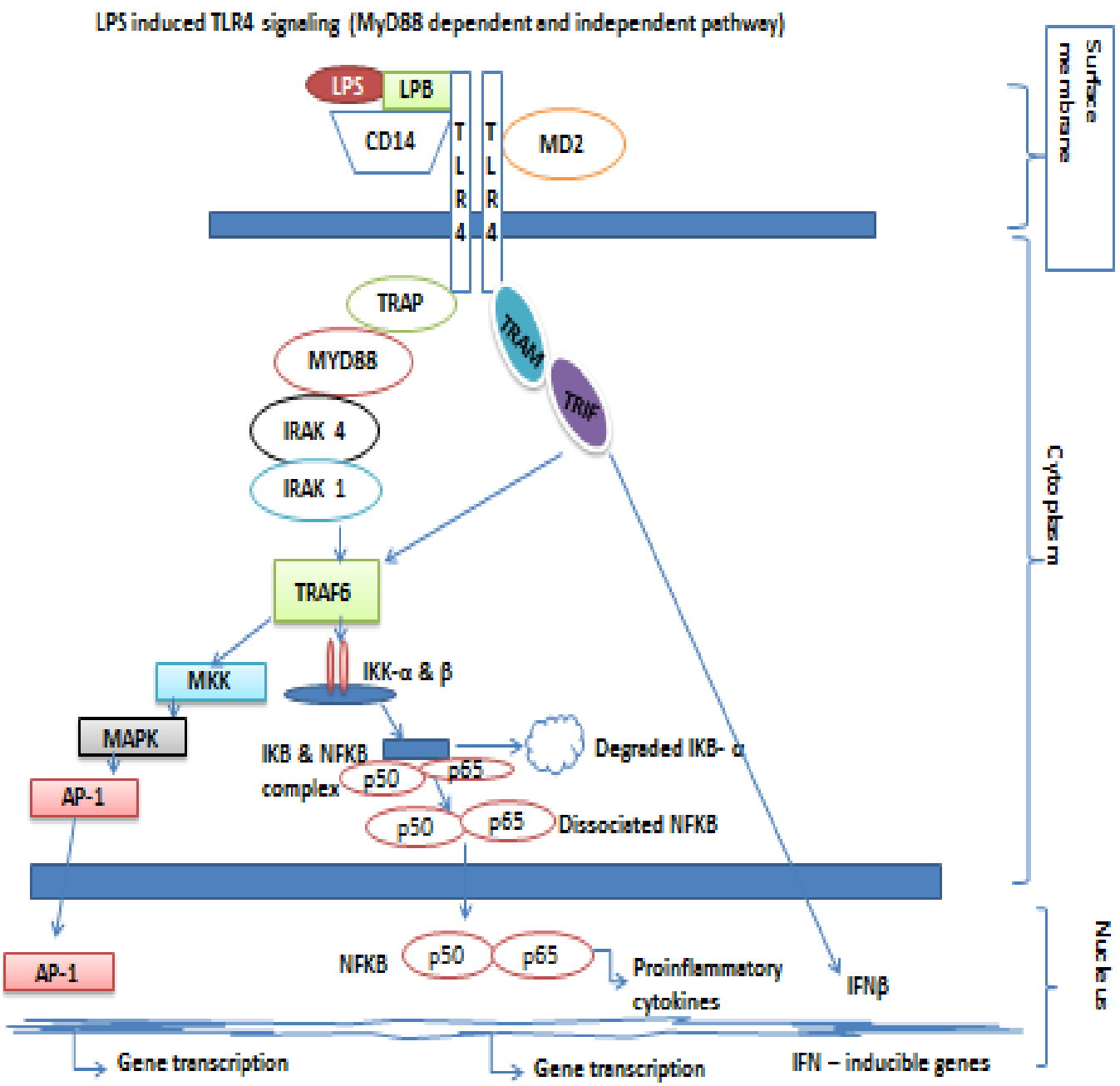


Figure 5: LPS induced TLR4 signaling (MyD88 dependent and independent pathway.

Modified from http://dx.doi.org/10.3389/fimmu.2014.00316

4. NUCLEAR FACTOR KAPPA –B (NF-KB) SIGNALING

The NF-kB is a family transcription factors that bind to discrete DNA sequences present in promoter and enhancer regions of different genes (Gilmore, 2006). There are five members of the NF-kB family in mammalian cells, which includes RelA (p65), RelB, C-Rel, p105 (NF-kB1 which is a precursor of p50) and p100 (NF-kB2, which is a precursor of p52). Formation of NF-kB homo - or hetero - dimers is required to regulate expression of genes that play critical roles in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, and stress responses (Gilmore, 2006). Under resting/un-stimulated conditions, the inactive NF-kB is present in the cytoplasm because of its interaction with IkB proteins. These IKB proteins prevent nuclear translocation of NFKB, thereby confining them in the cytoplasm.

Following TLR ligation and intracellular signaling such as seen following LPS stimulation, the major NF-kB family activated is a heterodimer composed of RelA (p65) and (p50) (Hayden *et al*., 2006). This heterodimer are present in the cytoplasm in an inactive form by interaction with protein inhibitors (IkB proteins) in unstimulated cells. Following stimulation, the IkB proteins are degraded by the IKK complex thus allowing the NFKB to translocate to the nucleus.

5. MITOGEN ACTIVATED PROTEIN KINASES (MAPKS)

MAPKs are important mediators of signal transduction and play a key role in the regulation of many cellular processes (Engelberg, 2004; Kyosseva, 2004). The MAPKs are serine/threonine kinases that are phosphorylated and activated by diverse stimuli such as proinflammatory cytokines, LPS, growth factors, and environmental stress. MAPKs are activated by a MAPK kinase (MAPKK) which in turn is activated by a MAPKK kinase (MAPKKK) (Wada *et al*.,

2004). Currently, four major MAPK pathways have been elucidated, including extracellular signal regulated kinase 1/2 (ERK1/2), c-Jun amino terminal kinase (JNK); p38 MAPK; and Big MAPK (BMK), also known as ERK5.

Upstream MKK3 and MKK6 are responsible for the phosphorylation of MAPKs proteins (Derijard *et al.,* 1995). Following activation, MAPKs proteins especially P38 and ERK1/2 plays a critical role in Th1 differentiation, IFN-γ production and production of proinflammatory cytokines (Newton and Holden, 2003; Pelaia *et al.*, 2005)

6. LIPOPOLYSACCHARIDE (LPS)

LPS has been shown to be a component of the outer membrane of gram-negative bacteria that strongly activate different mammalian cell types. LPS-responsive cells such as monocytes and macrophage rapidly gets activated after LPS interacts with circulating LPS-binding protein and CD14, a cell surface glycoprotein required for sensitive responses to LPS. Previous work has shown that LPS initiates several intracellular signaling events, which involve NF-kB activation with the production of pro - inflammatory mediators, such as interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-1 (IL-1), and tumor necrosis factor-alpha. LPS induced the intracellular signaling by the aid of Toll-like receptors (TLRs) which is a transmembrane receptor protein that relay the LPS- induced signals through the surface membrane of the cell.

7. CELL MEDIATED IMMUNITY

Cell mediated immunity is a type of adaptive immunity that does not involve B cell (antibody) response. In other words, T cells are the primary mediators of cell-mediated immunity. Three major effectors of T- cells that mediate cell-mediated immunity include CD4+ helper T- cells, CD8+ cytotoxic T- cells and regulatory T- cells. There are two major subsets of CD4+ helper T- cells that are classified based on the cytokines they produce. Th1 cells release IFN-γ, IL-12 and TNF and are known to enhance proinflammatory response that is critical for controlling intracellular pathogens. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and play a role in non-inflammatory-mediated immune response. In addition, Th2 cells play critical roles in helping B cells to produce antibodies such as IgG and IgE. As a consequence, Th2 cells are involved in allergic reactions including asthma.

8. CYTOKINES

Cytokines are small molecular weight water-soluble signaling proteins and glycoproteins with size less than 30kDa that are released by hematopoietic and non- hematopoietic cells (Holloway *et al*., 2002). They play a key role of regulating immune cells and hematopoiesis by mediating cell to cell communication through autocrine and paracrine pathways. The production of cytokines is limited under normal conditions; however during inflammatory conditions, cytokines are transiently induced in response to the causative stimuli (Abbas and Lichtman, 2003; Vilcek, 2003). Cytokines also act as a link between the immune system and the (inflamed) tissues (Yu *et al*, 2005), thus initiating host immune response (Wang *et al.*, 2000).

9. PROINFLAMMATORY CYTOKINES

As their name implies, these are cytokines that initiate and promote local or systemic inflammatory responses. Examples of these inflammatory cytokines include IL-12, TNF, IL-6, IL-8, etc. Due to their proinflammatory property, the production of these cytokines worsens several disease conditions such as fever, inflammation, tissue destruction, etc. Sometimes, excessive production of these cytokines results in severe and massive inflammation leading to cytokine storm and clinical conditions such as sepsis and septic shock. Proinflammatory cytokines are usually produced by innate immune and non-immune cells following their interaction with PAMPs expressed on invading pathogen.

13.1. IL-12

IL-12 previously referred to as T-cell differentiation factor (TCDF) or natural killer cell stimulatory factor (NKSF), is a 70kDa heterodimer protein that consists of two subunits (p35 and p40 subunits). P35 is expressed constitutively without any induction but at low levels while the expression of p40 is inducible under the influence of microbial stimulation, usually through recognition via the Toll-Like Receptors (TLR) on the antigen presenting cells (Watford *et al*., 2003). The production of IL-12 at the period of infection regulates innate responses and determines the type of adaptive immune responses. IL-12 plays a key role in the induction of Th1 cell-mediated immune responses against many pathogens in mice including *Leishmania* infections. This is because the binding of IL-12 to the IL-12 receptor (IL-12R) complex (IL-12Rβ1 and IL- 12Rβ2) on naïve T cells initiates intracellular signaling events leading to activation of the transcription factor (STAT 4) and differentiation and proliferation of naïve

CD4+ T cells into IFN-γ-producing cells (Watford *et al*, 2003).Furthermore, IL-12 inhibits Th2 polarization and its production of IL-4 and IL-10 cytokine.

Microbial induced signaling alone has been shown not to be strong enough to initiate the production of bioactive IL-12. Hence, the presence of additional activating signals are required to facilitate bioactive IL-12 production (Moser and Murphy, 2000; Trinchieri, 2003; Watford *et al*., 2003). During IL-12 signaling, cell to cell interaction occurs via CD40 co-receptor expressed on the surface of APC with its CD40 ligand (CD40L)) expressed on the surface T cells. The interaction of CD40-CD40L is a bi-directional event enabling signaling to both APC and T cells (Peng *et al*., 1996). In T cells, CD40-CD40L interaction induces T cell proliferation, IL-2 and IFN-γ production (Peng *et al*., 1996). In APCs, CD40-CD40L interaction results in augmented and enhanced bioactive IL-12 production.

13.2. IL-6

IL-6 was first identified following its cloning in 1980(Weissenbach *et al.*, 1980). Although all nucleated cell express IL-6, studies have shown that it is most commonly produced by monocytes / macrophages in response to endotoxin or LPS stimulation (Akira *et al*., 1990; Kato *et al*., 1990). The receptor for IL-6 (receptor IL-6R) exists as cell associated or in soluble form and is abundantly present on monocytes, hepatocytes and lymphocytes (Coulie *et al*., 1989). In response to infection, IL-6 can bind to either the cell-associated or the soluble IL-6R to initiate signaling (Mullberg *et al*., 11993). The binding of IL-6 to its receptor results in phosphorylation of Janus kinases (Jaks), which subsequently result in the phosphorylation of Signal Transducer and Activation for Transcription 3 (STAT-3) (Hirano *et al*., 1994).

13.3. TNF

Tumor necrosis factor was discovered in early 1960s by Aggarwal and his coworkers due to its major activity in inducing tumor regression in mice (Argawal *et al*., 1960). It is a glycoprotein induced by endotoxin /LPS, which induces tumor regression by causing hemorrhagic necrosis of sarcoma in mice (Carswell *et al.*, 1975). Agarwal isolated and identified 2 isoforms TNFs: lymphotoxin alpha / TNF-beta, and TNF /TNF- alpha. TNF alpha is 17kDa protein derived mainly from macrophage while TNF beta is derived from lymphocytes and is a 25KDa protein. Both TNF- alpha and TNF- beta bind to common surface receptors (TNFR1 and TNFR2) seen in most cell types. TNF is rarely detected in healthy individuals. However, high serum and tissue levels are detected in inflammatory, infectious and malignant conditions (Robak *et al*., 1998; Nurnberger *et al*., 1995).

In addition to its role in tumor regression, TNF has a powerful pro-inflammatory activity (Spriggs *et al*., 1987). The pro-inflammatory effects of TNF are explained on the basis of its effects on vascular endothelial cells and endothelial leukocyte interactions. TNF induces inflammation by locally acting on the vascular endothelial cells leading to the production of cyclooxygenase 2, which enhances extracellular expression of vasodilator PG1 causing vasodilatation (Mark *et al*., 2001), increased blood perfusion to site of inflammation / infection and recruitment of different leukocytes populations to the site of infection / inflammation. Cellular recruitment to the site is in response to the TNF on endothelial cells, thus inducing the expression of E-Selectin, ICAM-1, V-CAM1 (Pober *et al*., 1986; Munro *et al.*, 1989) and other chemokines such as IL-8 and MCP-1 (Rollins *et al*., 1990) on the surface of vascular endothelial cells.

14. LEISHMANIASIS

14.1. Epidemiology of *leishmaniasis*

Leishmaniasis is a spectrum of disease caused by several species of the obligate intracellular protozoan parasite belonging to the genus *Leishmania* (order Kinetoplastida). About twenty one different species of *Leishmania* has been identified as disease causing agent (Ruhland *et al*., 2007). It has been reported that about 1 – 1.5 million cases of cutaneous *Leishmaniasis* affect people annually (Desjeux, 2004). Recently, it has been shown that about 350 million people are at risk of getting infected (ref). *Leishmania* is transmitted through the bite of infected female sand flies of the genus Phlebotomous (Old World) and Lutzomyia (New World). Sand flies are abundantly seen in tropical and sub-tropical climates and inhabit areas such as desert or semi-arid regions.

14.2. Life cycle

Leishmania parasites have dimorphic life cycle alternating between the vector (sandfly) and the mammalian host. During a blood meal, infected female sand flies inject the infective promastigote to the host. Within the host, the promastigotes are phagocytized by phagocytic cells (Sacks and Sher, 2002). At an estimated period of 24 – 72 hours, promastigotes transform in the host cell into the intracellular/tissue form of the parasite referred to as amastigote. Amastigotes are small size (2-6μm) non motile form of *leishmania* with a vestigial flagellum. They are capable of surviving and replicating by simple division (maintain a neutral internal pH) in the harsh and highly acidic environment of the phagolysosome (Burchmore *et al*., 2001). When the cells are overwhelmed, they rupture to release the amastigotes that go on to infect other cells. A Sand fly becomes infected during blood meal after ingestion of infected macrophage cell

containing the amastigote. In the sand fly, the amastigotes are released in midgut during blood digestion and transform to promastigotes.

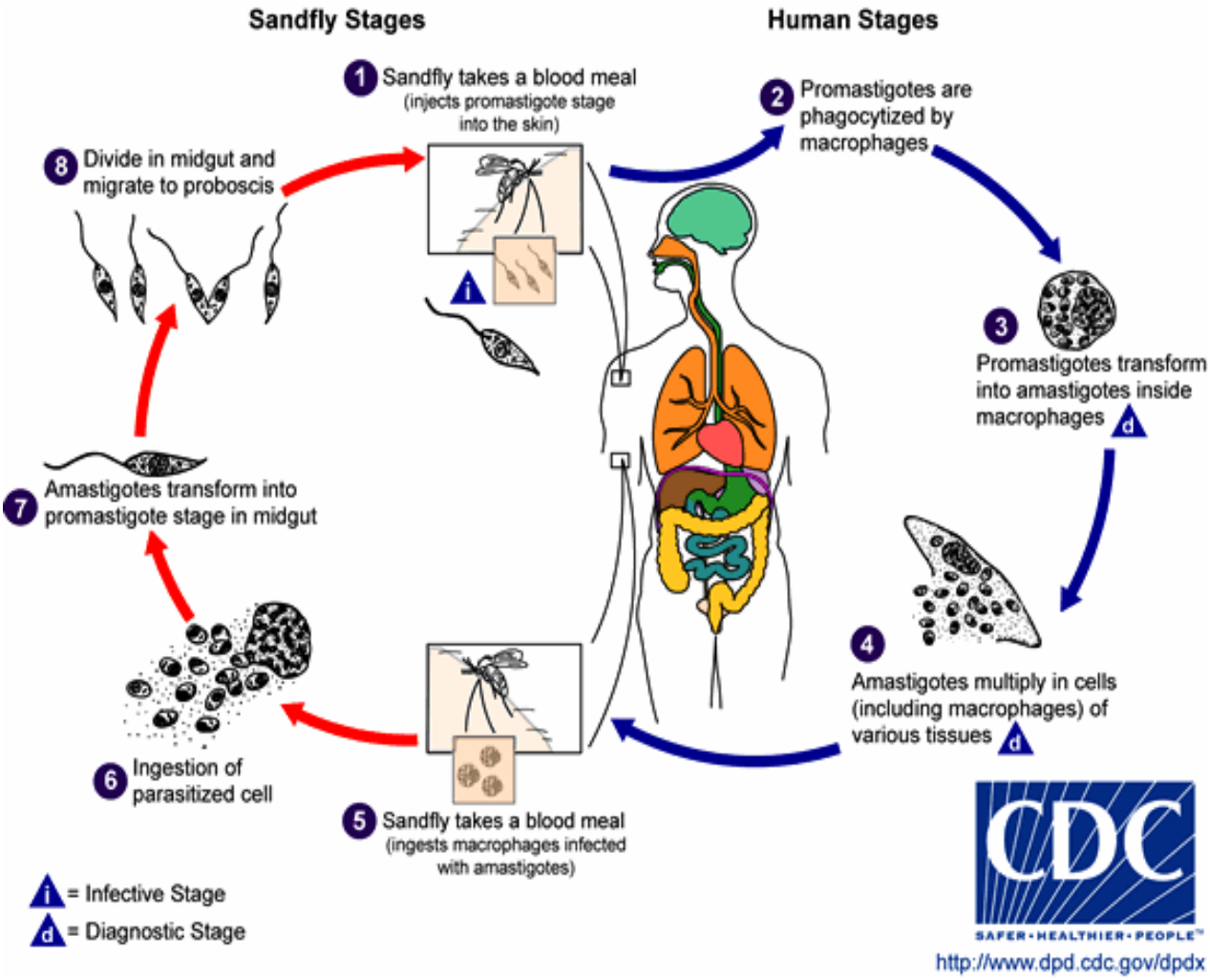


Fig 6: Life cycle of *Leishmania major*.

Public domain material fromhttp://www.dpd.cdc.gov/dpdx

14.3. Disease spectrum

Leishmaniasis occurs as a spectrum of clinical syndromes divided into cutaneous leishmanisis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). The epidemiology and clinical features are highly variable due to interplay of many factors such as parasite species, vectors, host and environment. Although all the different disease forms are clinically and equally important, this aspect of the literature review will focus on cutaneous leishmaniasis because this is the model that was investigated in this study.

14.4. Cutaneous *leishmaniasis* (CL)

CL is characterized by skin lesion development, which is usually self-limiting because it heals within few months of exposure (Berman, 2003). The causative agent of CL in the old world includes *L.major* and *L. tropicana* while in the new world, *L. mexicana, L. brazilliensis* and *L. panamensis* are responsible for the disease (Berman, 2003). CL is the most common form of *leishmaniasis* representing an average of 50 – 75% of all the new cases. In non-endemic areas, increased incidence of CL has been shown among veteran's workers. During the first gulf war, CL was shown to be low. However in year 2003 and 2004, CL was detected among 237 out of 20,000 US army soldiers returning from Operation Iraqi Freedom (OIF).

The first sign of CL is the development of small erythema at the site of infection by the infected female sand fly. This is followed by papule and nodule formation, which later ulcerates approximately within 2 weeks to 6 months to form a lesion resembling local cutaneous *leishmaniasis* (LCL) (Peter and Killick – Kendrick, 1987). Spontaneous healing has been shown to be associated with the development of an everlasting cutaneous scar as well as everlasting

immunity against the CL. The scar formed depending on the location on the body and its size might result in psychological trauma of the affected patient (Yanik *et al.*, 2004).

14.5. Murine model of cutaneous *leishmaniasis*

In humans, different species of *leishmania* are associated with distinct clinical diseases and the disease severity differs depending on the host involved (Bradley, 1974). This is also applicable to the mouse model of cutaneous *leishmaniasis* in which the outcome of the disease is dependent on the type of mouse strain involved and the nature of T cell response. While the C57BL/6 mice are resistant to *L. major* infection, the BALB/c mice are highly susceptible (Howard *et al.*, 1980). The resistance in the C57BL/6 mice is related to their ability to develop early Th1 immune response, which is important for inhibiting parasite proliferation. In contrast, the highly susceptible BALB/c develops an early Th2 response resulting in non-healing lesion and worsening of disease (Locksley *et al.*, 1987; Morris *et al*, 1993; Launois *et al*, 1999; Himmelrich *et al*., 2000).

14.6. Regulation of immunity to cutaneous *leishmaniasis*

Th1 immune response plays a key role in protecting mice against *leishmania* infection (Sacks and Noben – Trauth, 2002). The development of Th1 immunity involves the production of interleukin – 12 (IL-12) by the professional antigen presenting cells (APC) such as dendritic cell, production of interferon gamma (IFN-γ) by CD4+ T cells and macrophage activation by (IFN-γ) to make nitric oxide (NO) that kill intracellular parasites. Previous reports have shown that

susceptibility to *L.major* in mice is due to the induction of Th2 immune response. This is characterized by the failure of the APCs to synthesize IL-12 cytokine and/or inability of the CD4+ T cells to respond to the produced IL-12, which could be related to failure to express IL-12R. Alternatively, the presence of IL-4 or IL-10 were produced by several cells leads to the polarization of CD4+ T cells into Th2 cells that produce IL-10, resulting in macrophage deactivation, inability to kill parasites and susceptibility to *Leishmania major*. Previous studies on *L. major* infection using the murine models showed the direct evidence of the relevance of Th1/Th2 balance in regulating disease resistance and susceptibility respectively (Reiner and Locksley, 1995; Sacks and Noben- Trauth, 2002). Blockade or neutralization of IFN-γ (either by using antibodies or IFN-γ KO mice) abolishes resistance in the usually resistant C57BL/6 mouse model, confirming the importance of this cytokine as a key regulator in resistance and clearance of *L. major* (Howard *et al.,* 1980; Liew *et al*, 1982, Belosevic *et al.*, 1989; Heinzel *et al.,* 1989). In contrast, blockade of IL-4 signaling either with neutralizing anti-IL-4 antibodies or using IL-4 KO mice results in resistance in the usually susceptible mice. Furthermore, adoptive transfer studies further show that Th1 cells protect the highly susceptible mice against *L. major* infection while Th2 cells promote disease progression (Scott *et al.,* 1988). This protective role of IFN-γ is due to its ability to activate macrophages leading to the production of nitric oxide, a critical molecule that is important for intracellular parasite killing (Nathan et al., 1996).

15. NITRIC OXIDE (NO)

NO is a small biological signal and effector molecule that plays active role in intracellular and extracellular signaling. It has a short half-life of about few seconds; it is a very small molecule and hence has the potential to diffuse freely across membranes or within cells from its site of

production to the site of action. It has contributed a lot in diverse physiological and pathophysiological mechanisms in immune, cardiovascular, and nervous system of a living organism. Low level of NO regulates many physiological functions; however, pathological conditions have been associated with high concentration.

NO is produced from L- arginine using molecular oxygen, NADPH as electron donor, and heme, FMN, FAD as cofactors. The production is catalyzed by enzymatic groups called nitric oxide synthases (NOSs). The production of NO and L- citrulline is a two-step oxidative conversion of L-arginine to NO and L- citrulline through Nw – hydroxyl – L- arginine as an intermediate (Mayer and Hemmens, 1997; Alderton *et al*., 2001). There are three isoforms of NOS namely; neuronal nitric oxide synthase (nNOS, also called type 1 NOS) originating from neuronal tissues; inducible NOS / (iNOS, also called type 2 NOS) found in macrophages/ hepatocytes; and endothelial nitric oxide synthase (eNOS, type 3 NOS) which was discovered from endothelial cells. nNOs and eNOS are expressed constitutively without any induction but at low levels in a variety of cell types and tissue. Thus, they are collectively called constitutive NOS (cNOS). In contrast, iNOS expression is induced and the enzyme is not normally expressed at a significant level in cells and tissues (Wu and Morris, 1998) except during certain immunologic conditions such as production of inflammatory cytokines and bacterial endotoxins. The NOS isoforms can be induce under certain stimuli through transcriptional and translational mechanisms (Wu and Morris, 1998).

15.1. Regulation of NO synthesis by iNOS

The activity of iNOS in macrophages is regulated by intracellular events resulting from ligation of several cellular receptors such as Toll- like receptors by their ligands. For example, ligation of TLR4 by the bacterial endotoxin (LPS) leads to pro-inflammatory responses and nitric oxide production in monocytes and macrophages (Schroder *et al*., 2000). Similarly, ligation of IFN-γR on macrophages and monocytes by IFN-γ leads to the activation of iNOS and NO production through the Jak-STAT signaling pathway (Rao, 2000).

The level and amount of NO produced by cells are influenced by several factors including the animal species, the nature of the activating signal and presence of other inflammatory mediators that are known to affect Jak-Stat signaling pathway. For example, murine and bovine macrophages synthesizes large amount of iNOS (and hence NO) in response to cytokine stimulation. (Jungi*et al.*, 1996). NO is readily synthesized in mice with a wide variety of inflammatory or infectious conditions. However in vitro exposure to microbial products from gram negative bacteria (LPS) and cytokines such as interferon gamma, TNF- alpha, interleukin -1 are the most potent stimulators of macrophage for NO synthesis.

16. INTERFERON GAMMA (IFN-γ)

IFN-γ, also known as immune interferon is the major subtype of type 2 IFNs that binds to a receptor that is different from those that bind to type1 IFNS. The cytokine is also known as macrophage activating factor because it plays an important role in activating macrophage

physiologic activities including up regulating antigen processing, presentation of peptide to T-cells and stimulating the production of cytokines that have antimicrobial and anti-tumor effects. IFN-γ is mainly produced by Th1 subset of CD4+ T lymphocytes, CD8+ cytotoxic T-lymphocytes and NK cells. It can also be expressed by macrophages, B cells and dendritic cells (Freucht *et al*., 2001; Schroder *et al*., 2004), although the biological significance and relevance of IFN-γ expression by these cells remain unknown. The secretion of IFN-γ by NK cells is one of the major key early host defenses against infection. This is mediated via the production of two major cytokines (IL-12 and IL-18) by dendritic cells following recognition of pathogens. Aside from producing IL-12, dendritic cells also produce macrophage inflammatory protein -1 alpha (MIP-1 alpha), a chemokine that attracts NK cells to the site of infection.

16.1. Biological significance of IFN-γ

IFN-γ has been shown to have numerous immunomodulatory roles such as antiviral activity, inflammatory, and regulation of intracellular microbicidal functions of macrophages. For example, IFN-γ knockout mice or mice deficient in IFN-γR are highly susceptibility to bacterial and viral infections (Dalton *et al*., 1993; Van den Broeck *et al.*, 1995). In addition, it induces the expression of class I and II major histocompatibility complex (MHC) molecules on the surface of dendritic cells and enhances antigen processing (enhance the differentiation of naïve helper T – cell into Th1 or Th2 cells).The release of IFN-γ from Th1 subsets of CD4+ T cells leads to activation of macrophages, thus inducing cytokine production and the production of nitric oxide to facilitate the killing of intracellular parasites (Boehm *et al*., 1997; Stark *et al*., 1998; Schroder *et al*., 2004). However IFN-γ induced pro- inflammatory cytokine production in a severe chronic

inflammatory state is associated with immunopathologies (Canete *et al*., 2000; Ramji, 2009), Furthermore, IFN-γ regulates the cellular state by regulating cell proliferation, differentiation and apoptosis. Thus, IFN-γ prevents cellular proliferation by regulating the production of genes associated with cell death (e.g. Fas) (Dai *et al*., 1998).

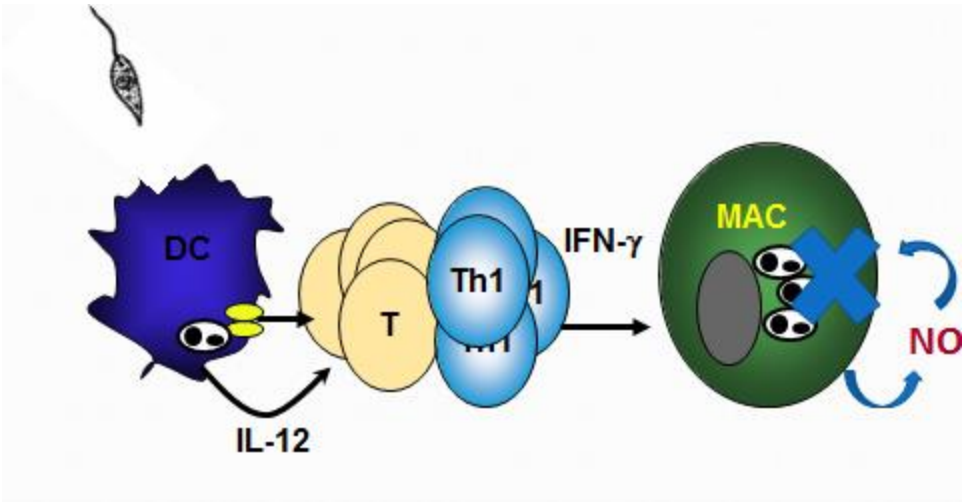


Fig 7. The known pathway leading to primary immunity to leishmania major in wild type mice following infection

17. JAK-STAT SIGNALING PATHWAY

Cell signaling is mediated by cytokine (IFN-γ) that binds to its specific receptors resident at the cell Surface. The interaction of IFN-γ with its receptor alters the receptors conformation and initiates intracellular signaling resulting in gene transcription, translation or post-translational modifications.

IFN-γ signals via the JAK - STAT signaling pathway. JAK - STAT pathway involves assembly of the IFN-γ receptors and activation of the family of Janus kinases and STAT that control the

transcription of target genes. As the IFN- γR1 (ligand - binding) binds with IFN-γ, they form a dimer and this induces them to get associated with two IFN-γR2 that are involved in signal transduction, thus assembly of these receptors induces the activation of JAK 1 and JAk 2 and phosphorylation of the tyrosine residue attached at the intracellular portion of IFN-γR1. This results in the recruitment of STAT1 which form homodimers and migrate to the nucleus and activate IFN -γ responsive genes. After signaling, the IFN-γR1 or ligand binding domain get internalized and dissociates, later recycle to the cell surface for another phase of signaling. Receptor internalization following signaling is mediated by Isoleucine – leucine sequence.

JAK 1 and 2 together with STAT1 are called signaling machinery and are used for signal transduction because the IFN-γR1 and IFN-γR2 lack intrinsic kinase/phosphates activity which could function to transduce signal. Cell signaling mediated by IFN-γ involves the binding of the IFN-γ to its IFN-γ receptor complex. The receptor complex involve the association of two ligand binding chains (IFN-γR1) and two non-ligand binding signal transducing chains (IFN-γR2). As the two IFN-γR1 (ligand - binding) binds with IFN-γ, they dimerise and get associated with two IFN-γR2 that are involved in signal transduction (Tau and Rothman, 1999), thus assembly of these receptors facilitates the activation of JAK 1 and JAk 2 and phosphorylation of the tyrosine residue attached at the intracellular portion of IFN-γR1 (Aaronson and Horvath, 2002; Leon and Zuckerman, 2005). The association of STAT1 with IFN-γR1 induces conformational change of IFN-γR1, though only JAK1 is required for the interaction of the two receptor chains, however, both JAK1 and JAK2 is important for any ligand-dependant conformational change (Krause *et al*., 2006).

Following IFN-γ binding to its receptors, it induces JAK 2 to undergo auto-phosphorylation. The activated JAK 2 in turn phosphorylates JAK 1 and the activated JAK1 has the capability to phosphorylate tyrosine residue seen on IFN-γR1, which subsequently serve as two adjacent docking sites for Src homology (SH) 2 domain of dormant STAT1. The dormant STAT1 dimer associated with IFN-γR1 gets phosphorylated at tyrosine residue 701 (Y701). Phosphorylated STAT1 homodimer dissociates from the receptor and undergo nuclear translocation where it binds to IFN-γ-activated site (GAS) elements located at the promoter of target genes to regulate the transcription of IFN-γ regulated genes (Platanias and Fish, 1999; Schroder *et al*., 2004). Though it has been shown that IFN-γ primarily signals via the STAT1 homodimer, however, it has been observed that STAT heterodimer (e.g. STAT1: STAT3) have also been found to activate transcription (Darnell *et al*., 1994). Thus, IFN-γ regulates its signaling pathway through a positive feedback loop associated with transcriptional activation of STAT1.

18. NEGATIVE REGULATORS OF IFN- γ SIGNALLING PATHWAY

IFN-γ signaling through JAK/STAT pathway has been shown to be negatively regulated by two major proteins: Suppressor of cytokine signaling (SOCS) protein and PTP (protein tyrosine phosphatase).

14.1. Suppressor of cytokine signaling (SOCS)

The suppressor of cytokine signaling (SOCS) proteins family is known as negative regulators of IFN-γ signaling. In mammals, eight SOCS family members have been identified to consist of SOCS1-7 (Hilton *et al*., 1998). Previous work has shown that induction of SOCS1and SOCS3

mRNAs occur under the influence of IFN-γ treatment (Starr *et al*., 1997). SOCS1 and SOCS3 have been implicated as inhibitor of tyrosine phosphorylation and nuclear translocation of STAT1 in response to IFN-γ.However SOCS1 displayed a much stronger inhibitory activity to IFN-γ signaling when compared to SOCS3 because SOCS 1 has higher affinity to Jak2 while SOCS 3 has a higher affinity for Tyk2. Thus, this suggests that SOCS1 and SOCS3 are the major inhibitors of IFN-γ-mediated Jak /STAT signaling pathways.

14.2. Control of signaling by SOCS 1 and 3 proteins

SOCS1-3 are induced specifically by the binding of IFN-γ cytokine with IFN-γ receptor and they function to inhibit signaling from IFN-γ receptor, thus inducing a negative feedback loop (Kamura *et al*; 2004). The major mechanism through which SOCS1 and SOCS3 has been shown to inhibit signaling include direct inhibition of Jak kinases activity by binding through their KIR domain to the JAK activation loop (Naka. *et al.,* 1997; Yasukawa *et al*; 1999).

II. RATIONALE

The binding of IFN-γ to IFN-γ receptor on macrophages results in their activation and production of pro-inflammatory cytokines and NO. Our preliminary data show that macrophages from PIP deficient mice produce significantly less NO and proinflammatory cytokine than their WT counterparts following IFN-γ priming. Whether PIP deficient macrophages are impaired in the expression of IFN-γ receptor and TLR4 is not known.

LPS and IFN-γ-stimulated bone marrow-derived macrophages from PIP deficient mice produce significantly less NO than those from WT mice. The production of NO and proinflammatory cytokines following macrophage activation is regulated by intracellular signaling events including MAPKs and STATs proteins.

III. HYPOTHESIS

The susceptibility of PIP deficient mice to *L.major* is related to impaired activation of their macrophages due to impaired expression of IFN-γ receptors and impaired intracellular signaling associated with defective MAPK and STAT phosphorylation.

IV OBJECTIVES

(1) Investigate the expression of IFN-γ receptors on primary macrophages (Peritoneal, splenic and bone marrow - derived) from WT and PIP deficient mice by flow cytometry.

(2) Assess for the phosphorylation of MAPKs (Erk and P38) and STATs (STAT1 and 3) in macrophages from WT and PIP deficient mice following stimulation with IFN-γ and LPS by Western blot.

V MATERIALS AND METHODS

1. ANIMAL HOUSING AND BREEDING

The generation of PIP deficient (PIP KO) mice has been previously described (Blanchard et al., 2009) Six to 8 weeks old female and male homozygous PIP KO mice (backcrossed > 9 generations into CD1 background) and wild-type (WT) CD1 mice were obtained from our in-house breeding colony managed by the Central Animal Care Services, University of Manitoba. The animals were housed and maintained in the specific-pathogen free environment at the University of Manitoba Animal care facility and the animals were used according to the guidelines stipulated by the Canadian Council for Animal Care. The mice were kept in plastic cages with wood chip bedding and fed rodent chow. Water and rodent chow were given *ad libitum.*

2. TISSUE COLLECTION

2.1. Isolation of bone marrow cells

Eight to twelve weeks old WT and PIP KO mice were sacrificed by cervical dislocation. The whole mouse was dipped in 70% of ethanol and the legs were excised, put in 15 ml centrifuge tubes (BD, VWR) containing 5 ml of RPMI-1640 (RPMI) media and kept on ice. The tibia and femur bones were separated from the skin and flesh using forceps and scissors. The two ends of the epiphyses were cut out and marrow cells were flushed off from the femur and tibia using a 5-ml syringe attached to 25-gauge needle containing complete RPMI media (RPMI supplemented with 10% heat-inactivated fetal bovine serum [FBS], 2 mM L- glutamine, 100U/ml penicillin/streptomycin and 5 x 10^{-5} 2 – mercaptoethanol). The bone marrow cells were pipetted up and down to make a single cell suspensions and the suspension was centrifuged at 4 degree

for 5 minutes at 1500 rpm. The supernatant was discarded and the pellet was cleared of red blood cells by resuspending in 3 ml of Ammonium-Chloride-Potassium (ACK) lysis buffer (0.155M of NH4CL, 0.01M of KHCO3, 0.1mM of EDTA dissolved in 1 litter of ddH20), and incubating for 5 minutes at room temperature. Additional 10 ml of complete RPMI was added to the cells and centrifuged at 4 ^{o}C for 5 minutes at 1500 rpm. The supernatant was discarded and the pellets were resuspended in 10 ml of complete medium. Cells were diluted 10 times in complete medium and further diluted 2 times in trypan blue (to make a final dilution of 1:20) and counted using a heamocytometer.

2.2. Isolation of splenic cells

Eight to twelve weeks old WT and PIP KO mice were sacrificed by cervical dislocation. The whole mouse was dipped in 70% ethanol and the abdomen was cut open through the right upper quadrant to expose the spleen. The spleens were excised and collected in 15 ml centrifuge tubes containing 5 ml complete RPMI media and kept on ice until processed. The spleens were homogenized using a sterile 15 ml tissue grinder (Fisher Scientific, Mississauga ON Canada) with 10 ml complete medium. The homogenate was passed through a sterile 70 µm cell strainer (VWR, Edmonton AB Canada) and the single cell suspension was centrifuged at 4 ^{o}C for 5 minutes at 1500 rpm. The supernatant was discarded and the pellet was resuspended in 3 ml ACK lysis buffer to remove contaminating red blood cell (as for bone marrow cells above). The cells were washed with 10 ml complete medium by centrifuging for 5 minutes at 1500 rpm. The supernatant was discarded and the pellets were resuspended in 10 ml of complete medium and counted as for bone marrow cells above.

3. DIFFERENTIATION OF BONE MARROW CELLS INTO BONE MARROW-DERIVED MACROPHAGES (BMDMS)

Five million bone marrow cells from WT and PIP KO mice were plated in tissue-culture treated petri (100 x 20mm Falcon cell culture dishes) in 10 ml BMDM medium consisting of 30% of L929 conditioned medium (LCCM) (which is a source of macrophage colony stimulating factor, M-CSF) and 70% complete medium. The cells were incubated at 37°C in a 5% CO_2 atmosphere and after 3 days, the cells received additional 10 ml of BMDM medium. On the 7^{th} day, the differentiated BMDMs, which were attached to the petri dishes, were washed with 10 ml of sterile PBS. The macrophages were then detached by gently scraping using a cell scraper. The cells were centrifuged at 4 ^{o}C for 5 minutes at 1500rpm; the pellets were resuspended in 10 ml complete media, counted using a haemocytometer and kept in ice until used.

4. DIFFERENTIATION OF BONE MARROW CELLS TO BONE MARROW DERIVED DENDRITIC CELLS (BMDDS)

Five million bone marrow cells from WT and PIP KO mice were plated in a petri dish in complete RPMI medium containing 20 ng/ml recombinant GM-CSF (Peprotech,Indianapolis, IN) The cells were incubated at 37°C in a 5% CO_2 atmosphere. After 3 days, the cultures were supplemented with extra 10 ml of complete RPMI medium containing 20 ng/ml rGM-CSF. On the 7^{th} day, non-adherent cells (immature dendritic cells) were gently detached by pipetting. The cells were then centrifuged at 4 ^{o}C for 5 minutes at 1500 rpm; the pellets were resuspended in 10

ml complete media and counted using a haemocytometer. Immature DCs were assessed by flow cytometry and the expression of CD11c was greater than 90%.

5. *IN VITRO* CELL CULTURES

BMDMs and BMDDCs from WT and PIP KO mice was diluted in a complete medium to a final concentration of 10^6/ml. One mililitre aliquots were seeded into a 24-well tissue culture plate (Falcon, VWR) and stimulated with or without LPS (1µg/ml, Sigma Mississauga, ON Canada), IFN-γ (40 ng/ml, BioLegend, San Diego, CA) or a combination of both LPS and IFN-γ. The cultures were incubated at 37°C in 5% CO_2 and after 48 hours, the supernatant fluids were collected and stored at -80 ^oC until used for ELISA.

6. SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

The levels of IL-12, IL- 6 and TNF cytokines in the supernatant collected from cell cultures were determined by sandwich ELISA. High binding ELISA plates (Immulon VWR, Mississauga, ON) were coated with primary antibodies (100 µl/well) at a concentration previously optimized in the lab (see table X below) in bicarbonate coating buffer (pH of 9.6) and incubated overnight (O/N) at 4 ^oC. The plates were washed 5 times with wash buffer (PBS containing 0.05% Tween-20, pH 7.4) and blocked for 2 hrs at 37 ^oC with blocking buffer solution (5% new calf serum in PBS, pH 7.4, 200 µl/well) to prevent non-specific binding. The plates were further washed 2 times with wash buffer and appropriately diluted recombinant cytokine standards (all from Preprotech) were titrated 2-fold serial dilution to generate standard curves (see Table X).

Samples were diluted in buffer and titrated serially in 2-folds and incubated overnight at 4 ^{o}C. The next morning, the plates were washed 5 times with washing buffer and 100 µl of biotinylated detection antibody at 2-4 µg/ml (Biolegend) was added to all wells. After 1-2 hr. incubation at 37 ^{o}C, the plates were washed 7 times, and streptavidin horseradish peroxidase (1:3000 dilution, BD Pharmagen, San Diego, CA) was added to all the wells and incubated for 30 minutes at 37 ^{o}C. The plates were then washed 10 times and ABTS substrate (Fisher, Life Technologies) was added to the plates. Plates were read at 405nm (Spectra Max) after the appropriate color development.

Table 1: List of cytokines, starting recombinant standard dilution, sample dilution and sensitivities of the sandwich ELISA

Cytokine	Standard (pg/ml)	Sample dilution	Sensitivity pg/ml
IL-12	5000	1:5	15.125
IL-6	4000	1:5	30.25
TNF	2000	1:5	30.25

7. SURFACE STAINING OF CELLS WITH FLUOROCHROME-CONJUGATED ANTIBODY

One mililiter of PBS containing 5 x 10^5 BMDMs or splenic cells were added into flow cytometry tubes (Falcon, BD Biosciences, San Diego, CA). The cells were then washed with 1 ml of fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.1% newborn calf serum and 0.1% sodium azide) by spinning in a centrifuge (Eppendorf) for 5 minutes at 1500rpm at 4 ^oC. Thereafter, the supernatant fluids were discarded and the excess fluids were blotted out using paper towel. The cells were then incubated with Fc receptor blocker (50 µl mouse IgG hybridoma supernatant for 5 minutes on ice, washed and then incubated with 20 µl (containing 0.01-0.05 mg) of fluorochrome-labeled antibodies against the following surface molecules: CD11b, (APC), CD11c (APC), IFN-γR (PE) and TLR4 (PE). After 25-30 min. incubation on ice, the cells were washed, resuspended in 300 µl FACS wash buffer and acquired using FACS Canto II (BD).

8. FLOW CYTOMETRY ANALYSIS

Flow cytometry analyses were carried out according to standard procedures. Briefly, stained cells were acquired using FACS Canto II (at least 100,000 events). The splenic cells were first gated on live cells and then on $CD11b^+$ cells (which is a marker of macrophages). Thereafter, the expression of IFN-γR and TLR4 by $CD11b^+$ cells were then analyzed and plotted as a histogram over isotype control antibody. To analyze the expression of these receptors on splenic dendritic cells, live cells were gated on $CD11c^+$ a cell (which is a marker of dendritic cells) and their

expression of the expression of IFN-γR, TLR4 and TLR2 was then analyzed. Similarly, the BMDMs and BMDCs were acquired and live events were further gated for CD11b+ and CD11c+ cells, respectively, and assessed for expression of IFN-γR and TLR4. The analyses were performed using Flowjo software (Treestar, Ashland, OR).

9. STIMULATION OF BMDMS FOR WESTERN BLOTTING

Two mililiter of incomplete RPMI media (without serum) containing 2 x 10^6 BMDMs from WT and PIP KO mice were added in small petri dishes (35 x 10mm style, Becton diskinsonlabware) and incubated overnight at 37 ^oC in a 5% $C0_2$ atmosphere to synchronize the cells. The next morning, the cells were washed and the media was replaces with complete RPMI medium and left unstimulated or stimulated with LPS (1µg/ml, Sigma), IFN-γ (40 ng/ml, BioLegend) or a combination of both LPS and IFN-γ. At different times (0, 15, 30 60 and 120 minutes), the media was removed and the cells were lysed with a combination of NP- 40 lysis buffer and protease inhibitors (sodium orthoVanadate, and PMSF).

10. DETERMINATION OF PROTEIN CONCENTRATION

The cell lysates were and centrifuged at 12,000 rpm for 10 minutes to remove cell debris. The supernatant was collected and protein concentration in the cell lysate was determined using Bicinchoninic acid assay (BCA, Sigma) according to the manufacturer's suggested protocol. The optical absorbance was measured at 405nm in a plate reader (Spectra Max, Sunnyvale, CA). The lysates were stored at -80 degrees for Western blot analysis.

11. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) FOR MAPKS, STATS AND SOCS PROTEIN

Frozen cell lysates from WT and PIP KO mice were thawed and the desired amount of each sample was mixed with 6x loading dye and lysis buffer (to make up the volume required for loading). The tubes were first centrifuged at 1200 rpm for 2 minutes, heated at 100 ^{o}C for 5 minutes to denature the proteins and then centrifuged again for additional 2 minutes. They were then loaded (5 μl/well) unto the wells together with standard size protein marker (Precision Plus Protein Standard Dual Color, BioRad laboratories Inc., ON, Canada) containing proteins of molecular weight size ranging from 10 – 250kDa. The samples were run in SDS-PAGE consisting of 10% separating gel and 4% stacking gel using Tris-glycine buffer system as the running buffer. Electrophoresis was performed at 90 volts for 20 minutes and later changed to 120 volts for 2 hours.

12. WESTERN BLOT ANALYSIS FOR ASSESSING THE PHOSPHORYLATION OF STATS, MAPKS, SOCS PROTEIN

Following electrophoresis, the proteins were transferred onto a polyvinylidenediflouride (PVDF) membrane (BioRad) at 90 volts for 90 minutes using the transfer buffer (10 x Running buffer, dH20, and methanol). The blotted PVDF membranes were blocked with 5% bovine serum albumin (BSA) in Tris Buffered Saline with Tween® 20 (TBXT) and incubated for 1hr at room temperature or overnight at 4 ^{o}C. Thereafter, the membranes were incubated overnight with primary antibodies (P-STAT1, P-ERK, P-P38 or P-NFκβ) at a dilution of 1:1000 in blocking solution. The membranes were washed 4 times at 5 minutes interval with Tris Buffered Saline with Tween® 20 (TBXT), and then incubated for 1 hr at room temperature with secondary

antibody (horse raddish peroxidase (HRP) conjugated goat anti-rabbit IgG, BioRad) at a dilution of 1:3000 in blocking solution. The secondary antibodies were washed off with TBST for 4 times at 5 minutes intervals and developed using ECL kit (GE Healthcare). ECL kit utilizes the reaction of the horseradish peroxidase (HRP) conjugated to the secondary antibody with substrate (consisting of luminal and peroxide solution) to detect antigens immobilized onto the PVDF membrane. The emitted signals were acquired at different times (1, 5, 10, 30 sec) using the ECL select developing machine (GE Healthcare). Following the detection of the phosphor-proteins, the membranes were stripped using stripping buffer (1 molar glycine, 10% SDS, Tween 20, dH20). They were then reprobed with anti-body against total STAT1, ERK, P38 or NFκβ as done for the phospho proteins.

13. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) FOR PIP EXPRESSION

Frozen cell lysates from WT and PIP KO mice were thawed and the desired amount of each sample was mixed with 6x loading dye and lysis buffer (to make up the volume required for loading). The tubes were first centrifuged at 1200 rpm for 2 minutes, heated at 100 ^{o}C for 5 minutes to denature the proteins and then centrifuged again for additional 2 minutes. They were then loaded (5 µl/well) unto the wells together with standard size protein marker (Precision Plus Protein Standard Dual Color, BioRad laboratories Inc., ON, Canada) containing proteins of molecular weight size ranging from 10 – 250kDa. The samples were run in SDS- PAGE consisting of 15% separating gel and 4% stacking gel using Tris-glycine buffer system as the running buffer. Electrophoresis was done at 90 volts for 20 minutes and later changed to 120 volts for 2 hours.

14. WESTERN BLOT ANALYSIS FOR PIP EXPRESSION

Following electrophoresis, the proteins were transferred onto a polyvinylidenediflouride (PVDF) membrane (BioRad) at 90 volts for 90 minutes using the transfer buffer (10 x Running buffer, dH20, and methanol). The blotted PVDF membranes were blocked with 5% milk in Tris Buffered Saline with Tween® 20 TBXT and incubated for 1hr at room temperature or overnight at 4 ^{o}C. Thereafter, the membranes were incubated overnight with primary antibodies (Anti – mouse PIP antibody) at a dilution of 1:500 in blocking solution. The membranes were washed 4 times at 5 minutes interval with TBXT, and then incubated for 1 hr at room temperature with secondary antibody (horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, BioRad) at a dilution of 1:3000 in blocking solution. The secondary antibodies were washed off with TBST for 4 times at 5 minutes intervals and developed using ECL kit (GE Healthcare). ECL kit utilizes the reaction of the horseradish peroxidase (HRP) conjugated to the secondary antibody with substrate (consisting of luminal and peroxide solution) to detect antigens immobilized onto the PVDF membrane. The emitted signals were acquired at different times (1, 5, 10, 30 sec) using the ECL select developing machine (GE Healthcare).

15. DENSITOMETRIC ANALYSIS

Densitometry was performed using the band analysis tools of the Image Lab software version (Bio- Rad). The tool is used to select and measure the background and this is subtracted from the density of the bands in all the film. The image software interprets the data and T- test was performed to determine the significance of differential density bands.

16. DATA ANALYSIS

Two way analysis of variance (ANOVA) was used to compare mean and standard deviation (SD) between the two groups. Bonferroni *t* test was used where there was significant difference in ANOVA. Difference were considered significant when $p < 0.05$.

VI RESULTS

1. DEFICIENCY OF PIP DOES NOT AFFECT THE EXPRESSION OF IFN-γR AND TLR4 ON BMDMS AND SPLENIC MACROPHAGES

The binding of IFN-γ and LPS to IFN-γR and TLR4 respectively on macrophages results in their activation and production of proinflammatory cytokines (ref). Our preliminary data showed that macrophages from PIP deficient mice produce significantly less nitric oxide and proinflammatory cytokine than their WT counterparts following IFN-γ and LPS priming. The reason for this is currently unknown. We therefore hypothesized that deficiency of PIP results in impaired IFN-γR and TLR4 expression on macrophages. Macrophages were differentiated from bone marrow cells from WT and PIP KO mice *in vitro*, surface stained with fluorochrome-conjugated antibody against IFN-γR and TLR4, and the expression of these receptors was assessed by flow cytometry. In addition, we also assessed and compared the expression of IFN-γR and TLR4 on primary (splenic macrophages) cells directly taken from sacrificed mice *ex vivo*. We found that BMDMs and splenic macrophages from WT and PIP KO mice express similar levels of IFN-γR and TLR4 (Fig.8A and B).

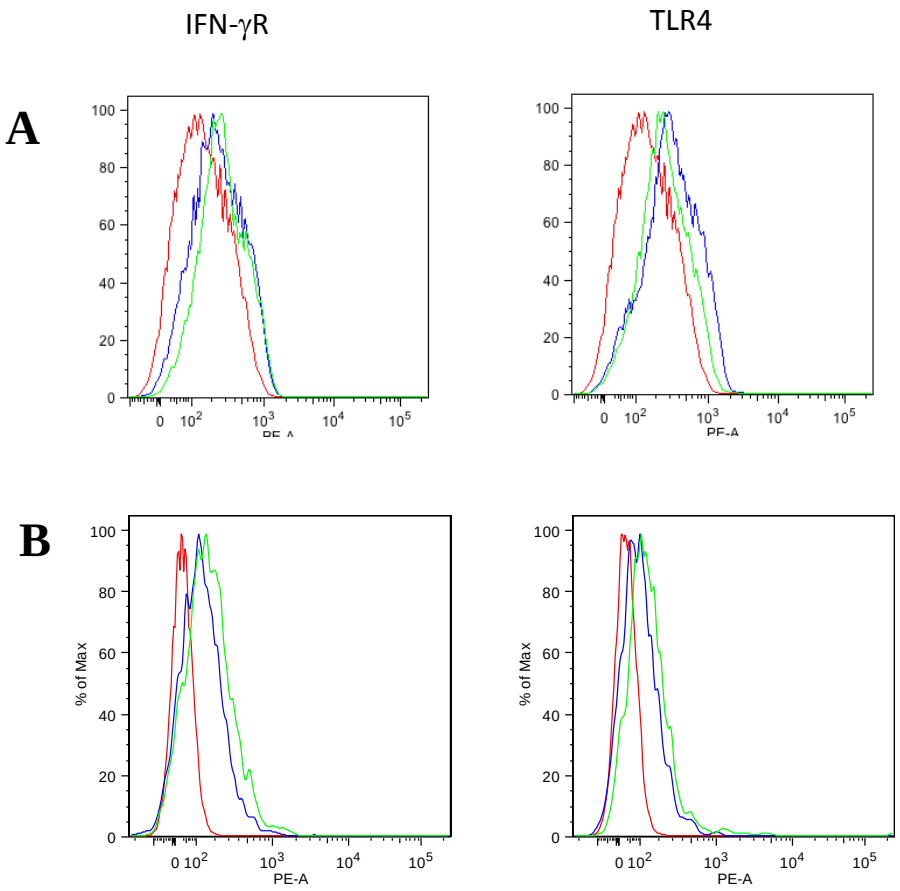


Fig.8

BMDMs and Splenic macrophages from WT and PIP KO mice express similar levels of IFN-γR and TLR4:

One milliliter of PBS containing 5 x 10^5 BMDMs or splenic cells was added to flow cytometry tubes. The cells were then washed with 1 ml of FACS buffer by spinning in a centrifuge for 5 minutes at 1500rpm at 4 ^oC. Thereafter, the supernatant fluids were discarded and the excess fluids were blotted out using paper towel. The cells were then incubated with Fc receptor blocker for 5 minutes on ice, washed and then incubated with 20 µl of fluorochrome-labeled antibodies against the following surface molecules: CD11b, (APC), CD11c (APC), IFN-γR (PE) and TLR4 (PE). After 25-30 min. incubation on ice, the cells were washed, re-suspended in 300 µl FACS wash buffer and acquired using FACS Canto II (BD). **A:** The BMDMs were acquired and live events were further gated for CD11b+ (which is a marker of macrophages) and assessed for expression of IFN-γR and TLR4. **B:** Similarly, the splenic cells were first gated on live cells and then on $CD11b^+$ cells. Thereafter, the expression of IFN-γR and TLR4 by $CD11b^+$ cells were then analyzed and plotted as a histogram over isotype control antibody. The analyses were performed using Flowjo software.

2. IMPAIRED PRODUCTION OF PROINFLAMMATORY CYTOKINES BY BMDMS AND BMDDC FROM PIP KO MICE FOLLOWING STIMULATION WITH IFN-γ AND LPS.

It is well established that priming of BMDMs and BMDDCs with IFN-γ is associated with the production of proinflammatory cytokine (IL-12 and IL-6). Our preliminary data show that BMDDCs from PIP deficient mice produce significantly less proinflammatory cytokine than their WT counterparts following IFN-γ priming. However, this study did not show if BMDMs from PIP deficient mice also showed impaired production of proinflammatory cytokine compared to their WT counterpart mice. BMDMs and BMDDCs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of LPS, IFN-γ, and LPS + IFN-γ for 48hr and the production of IL-6 and IL-12 in the supernatant fluids were determined by ELISA. In all experiment, unstimulated cells (medium) served as control. Consistent with the observations in BMDCs, we found that production of IL-12 and not IL-6 by the BMDMs from PIP KO mice was significantly impaired compared to their WT counterparts (Fig. 9C and D) (**$p < 0.01$).

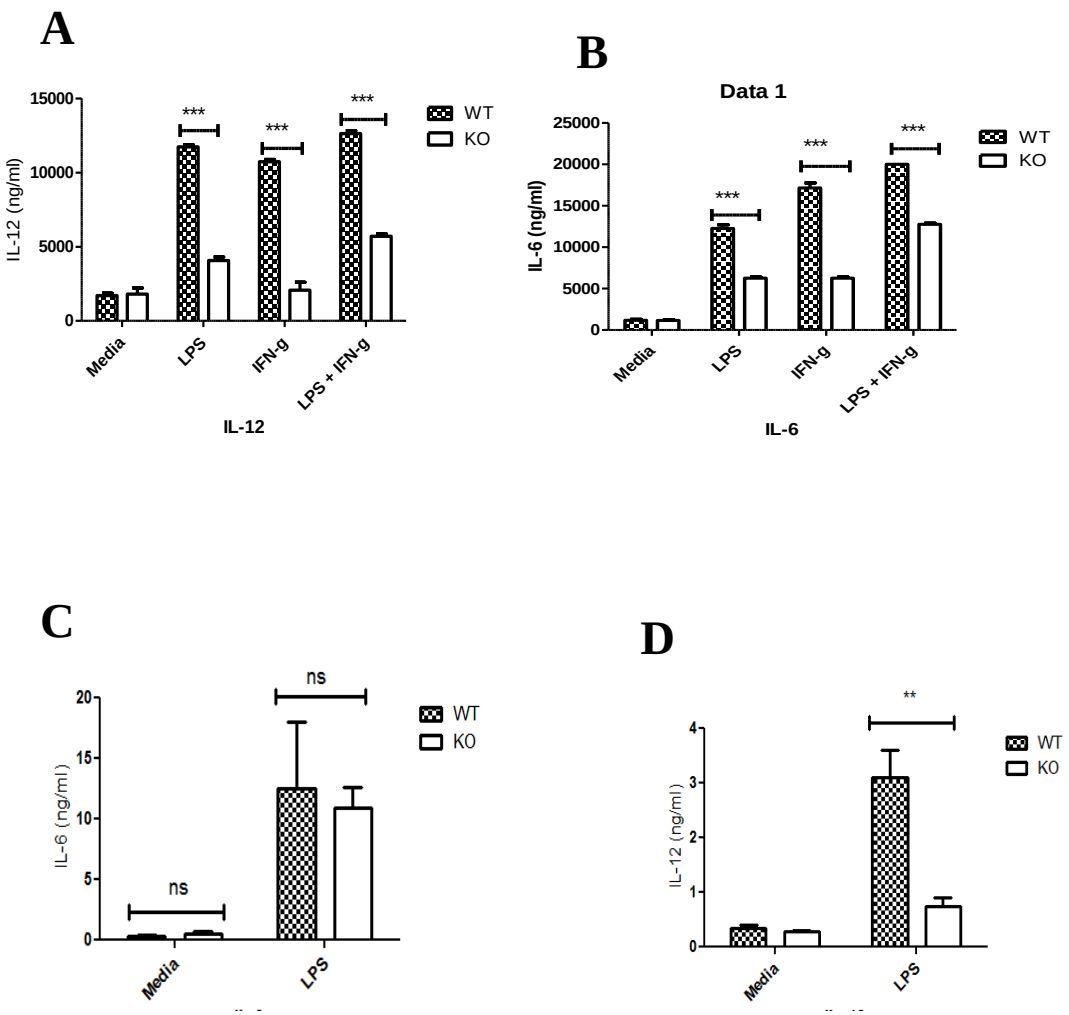


Fig. 9

Production of IL-12, IL-6 by the BMDDCs and BMDMs from PIP KO mice is impaired compared to their WT counterparts:

BMDDCs (**A and B**) and BMDMs **(C and D)** from WT and PIP KO mice were stimulated *in vitro* with various concentrations of LPS, IFN-γ, LPS + IFN-γ for 48 hr and the production of IL-12 (**A and D**), IL-6 (**B and C**) were determined by ELISA. In all experiments, unstimulated cells (medium) served as negative control, n = 3 (**p <0.01; ***p < 0.001)

3. IMPAIRED PHOSPHORYLATION OF STATs AND MAPKs PROTEIN FROM BMDMs IN PIP KO MICE FOLLOWING STIMULATION WITH IFN-γ AND LPS.

The production of NO and proinflammatory cytokines by activated macrophages is regulated by intracellular signaling events including phosphorylation of MAPK and STAT proteins. Our preliminary studies show that following LPS or IFN-γ activation, macrophages from PIP deficient mice produce significantly less NO and proinflammatory cytokines than those from WT mice. Since the expression of IFN-γR and TLR4 is not impaired in cells from PIP deficient mice, we hypothesized that deficiency of PIP might affect intracellular signaling pathways, particularly MAPKs and STATs phosphorylation in macrophages. BMDMs from wild type and PIP KO mice were stimulated *in vitro* with varying concentrations of LPS, IFN-γ or both and at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of Erk and p38 (MAPKs), STAT-1and STAT-3 (STATs) by Western blot as was done previously (Kuriakose *et al*, 2014). We observed that deficiency of PIP leads to impaired phosphorylation of Erk, P38 (MAPKs) and STAT3 except STAT 1 proteins in macrophages following stimulation with IFN-γ and LPS (Fig. 10 -1 5).

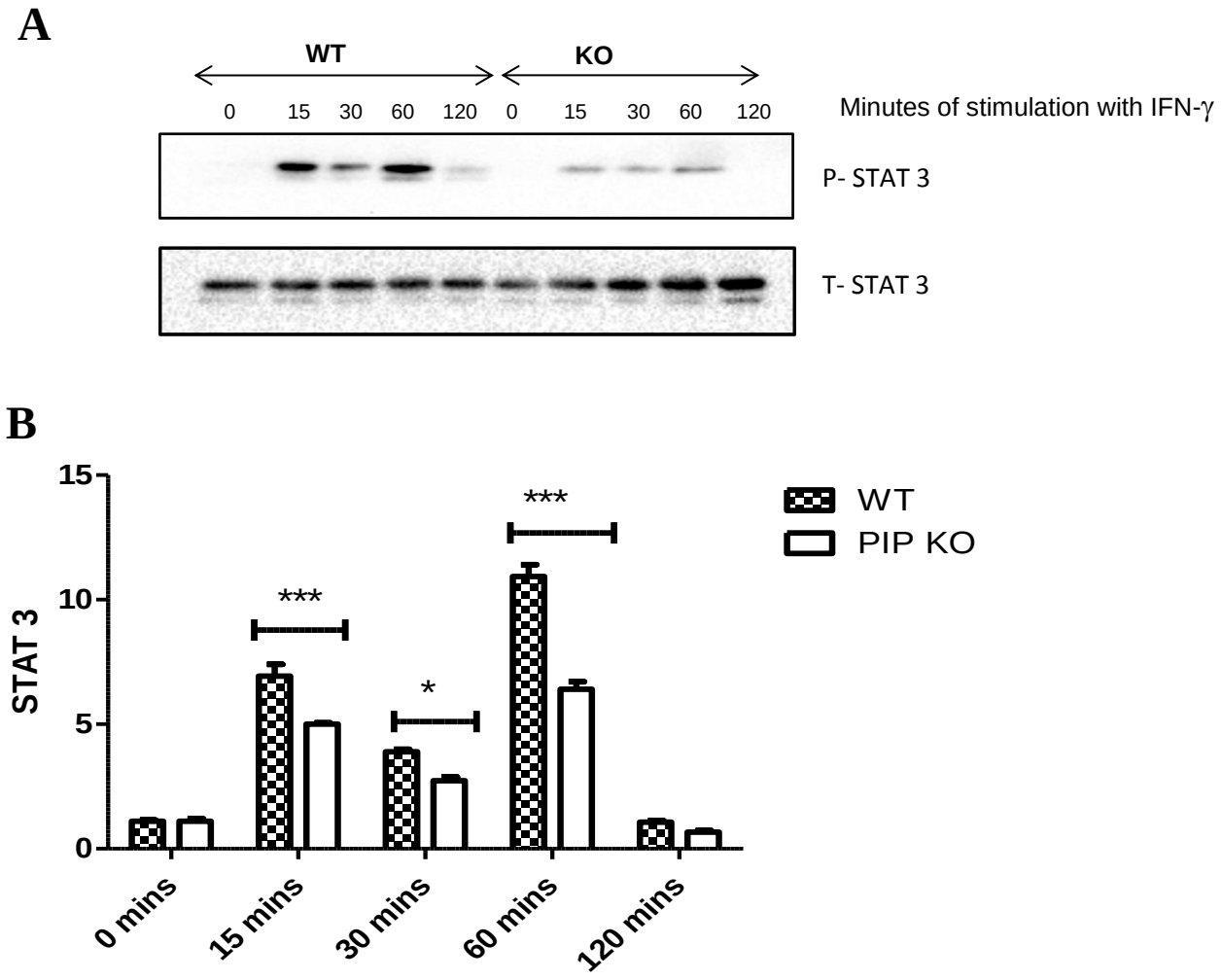


Fig. 10

Impaired phosphorylation of STAT 3 protein in BMDMs from PIP KO mice stimulated with IFN-γ

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of STAT-3 by Western blot. **B**: Analysis through densitometry of western blot data probed against phospho - STAT 3 and normalized against total STA T 3 levels that represent protein loading, n = 3. (**p* <0.05; ****p* <0.001).

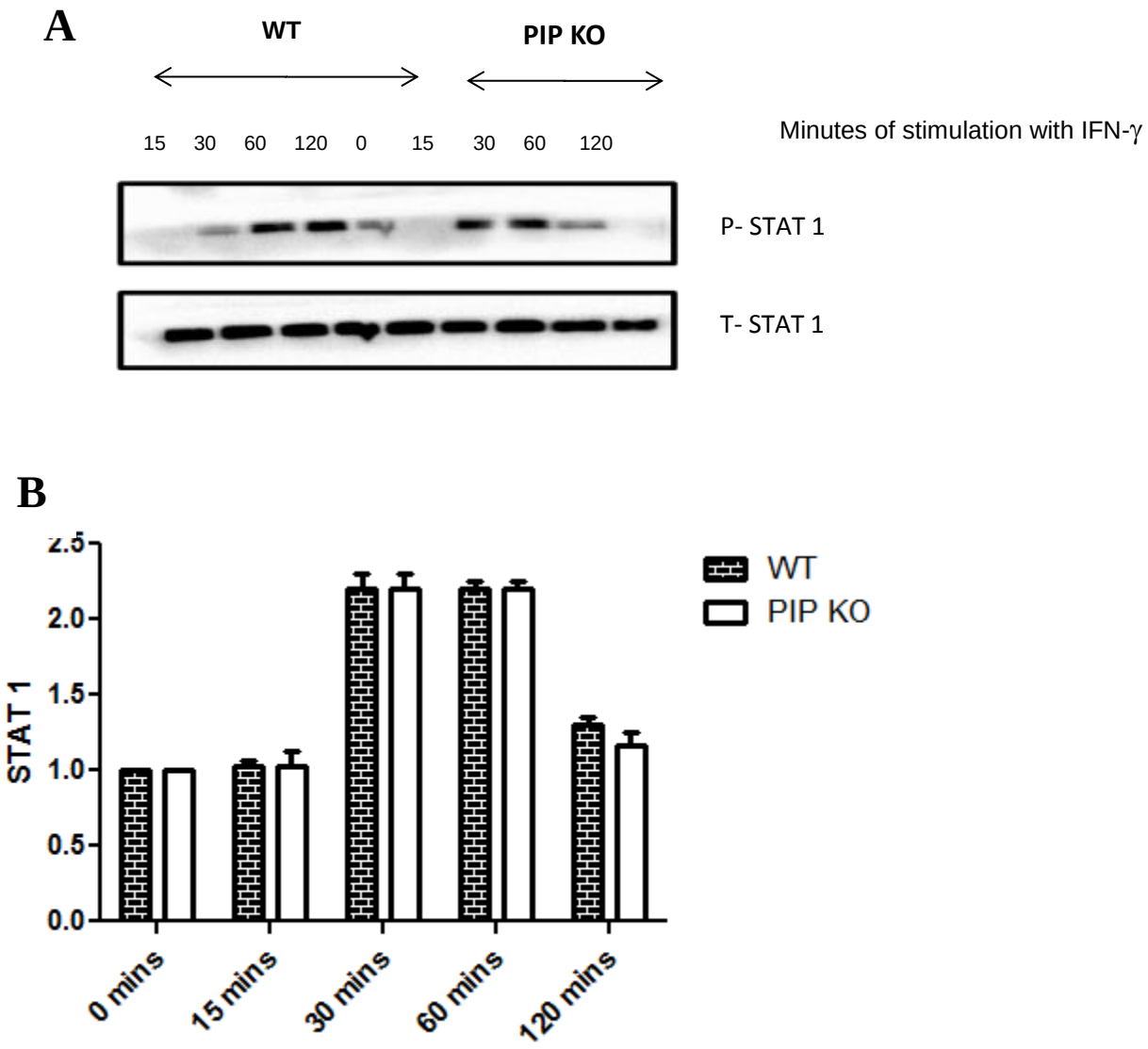


Fig. 11
Deficiency of PIP does not impair phosphorylation of STAT 1 protein in BMDMs from PIP KO mice stimulated with IFN-γ

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of STAT-1 by Western blot. **B**: Analysis through densitometry of western blot data probed against phospho - STAT 1 and normalized against total STA T 1 levels that represent protein loading, n = 3.

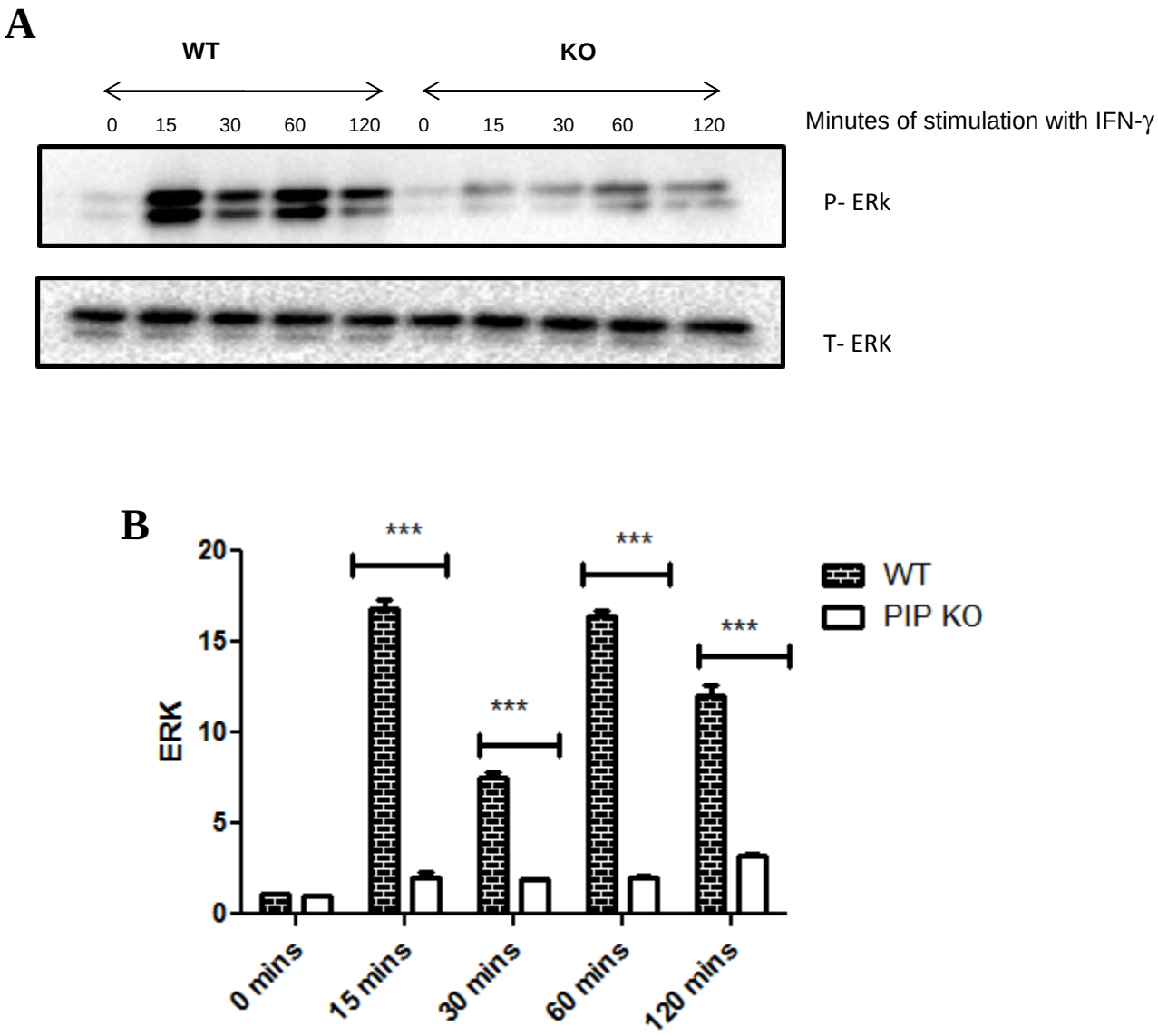


Fig. 12

Impaired phosphorylation of ERK protein in BMDMs from PIP KO mice stimulated with IFN-γ

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of ERK by Western blot. **B**: Analysis through densitometry of western blot data probed against phospho - ERK and normalized against total ERK levels that represent protein loading, n = 3. (***p <0.01)

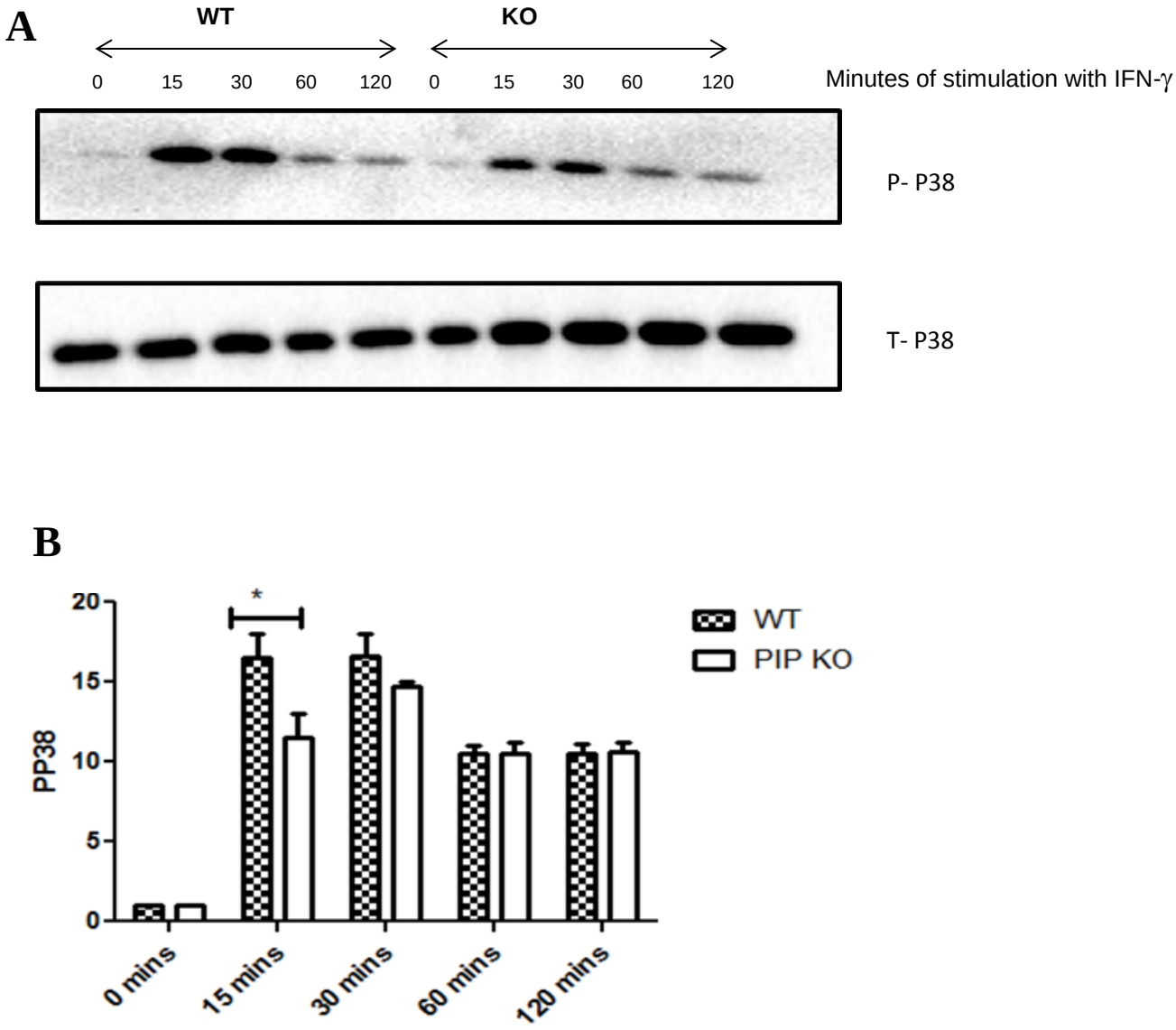


Fig. 13

Impaired phosphorylation of P38 protein in BMDMs from PIP KO mice stimulated with IFN-γ- A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of P38 by Western blot. **B**: Analysis through densitometry of western blot data probed against phospho – p38 and normalized against total p38 levels that represent protein loading, n = 3. (*p <0.05).

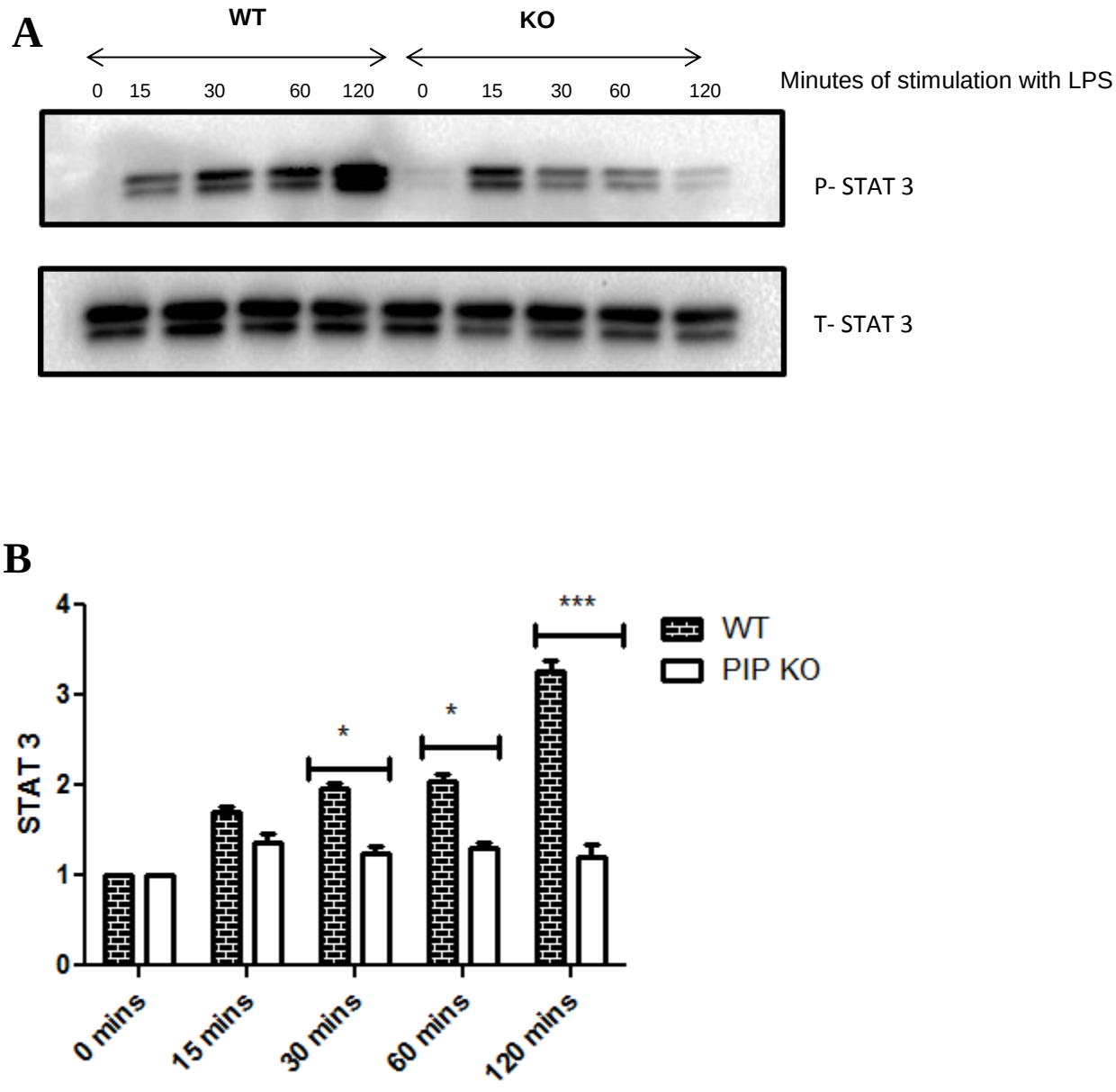


Fig. 14

Impaired phosphorylation of STAT-3 proteins in BMDMs from PIP KO mice stimulated with LPS:

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of LPS at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of STAT 3 by Western blot. **B**: Analysis through densitometry of western blot data probed against phospho – STAT3 and normalized against total STAT3 levels that represent protein loading n = 3. (**p* <0.05; ****p* <0.001).

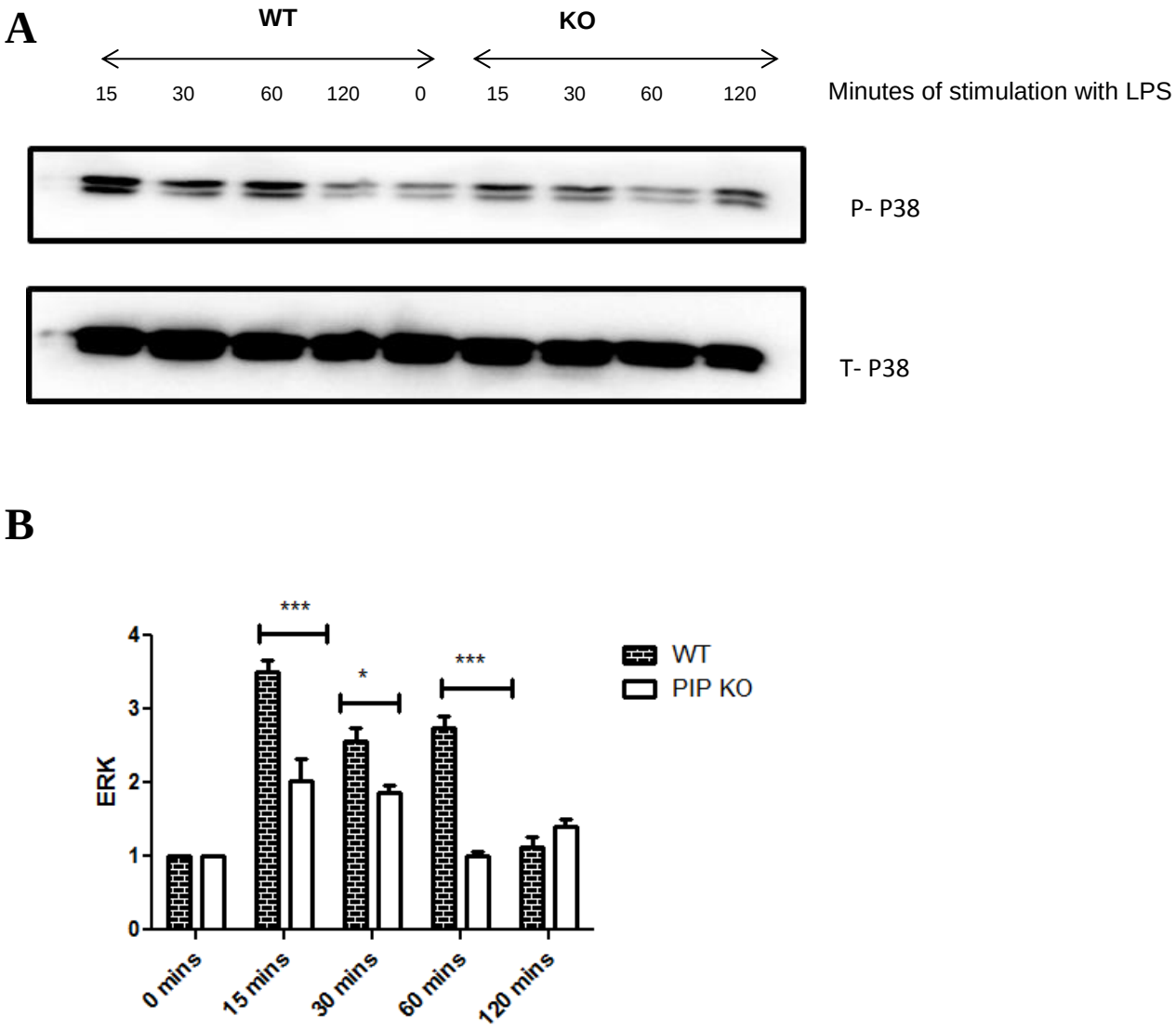


Fig. 15

Impaired phosphorylation of ERK proteins in BMDMs from PIP KO mice stimulated with LPS:

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of LPS at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of ERK by Western blot. **B**: Analysis through densitometry of western blot data probed against phospho – ERK and normalized against total ERK levels that represent protein loading, n= 3. (**p* <0.05; ****p* <0.01).

4. IMPAIRED PHOSPHORYLATION OF NUCLEAR FACTOR-KB (NF-KB) PROTEIN FROM BMDMS IN PIP KO:

The production of NO and proinflammatory cytokines by activated macrophages is regulated by NF-κB, a master transcription factor whose phosphorylation regulates proinflammatory cytokines gene expression in macrophages. Previous reports show that phosphorylation of MAPKs and STATs proteins leads to activation of the NF-κB pathway, leading to its translocation into the nucleus to activate NF-κB responsive genes. Because we found that deficiency of PIP impairs phosphorylation of MAPKs and STATs, we hypothesized that PIP deficiency would also impair NF-κB activation. We found significantly lower phosphorylation of NF-KB (p65) subunit in BMDMs from PIP KO mice stimulated with LPS compared to their WT counterparts (Fig. 16).

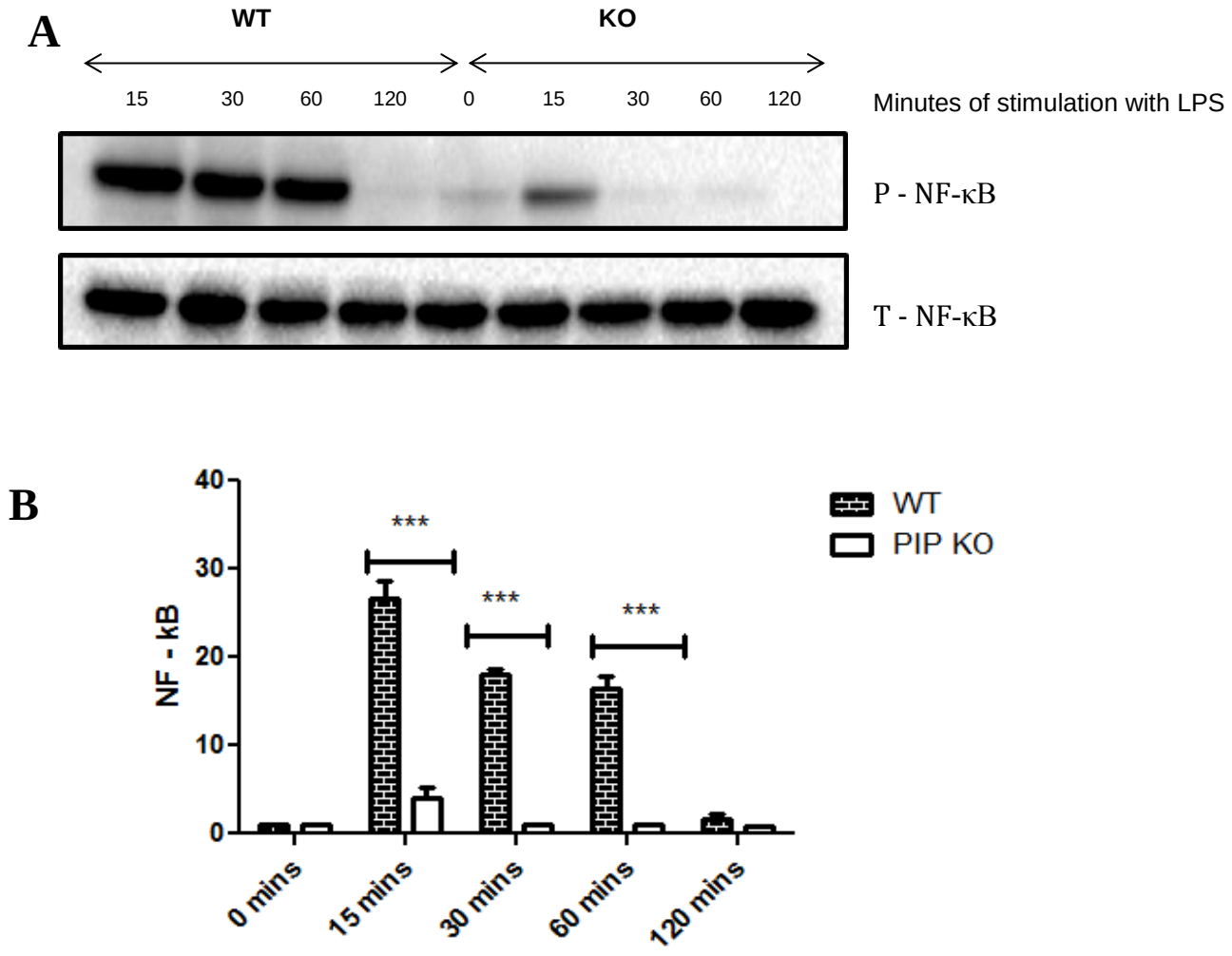


Fig. 16

Impaired phosphorylation of NF-κB protein in BMDMs from PIP KO mice stimulated with LPS

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for phosphorylation of NF-κB protein by Western blot. **B:** Analysis through densitometry of western blot data probed against phospho – NF-κB and normalized against total NF-κB levels that represent protein loading; n= 3. (***p <0.001).

5. THE EXPRESSION OF SUPPRESSOR OF CYTOKINE SIGNALING 1 AND 3 (SOCS 1 AND 3) PROTEIN IS SIGNIFICANTLY UPREGULATED IN BMDMs FROM PIP KO MICE

So far, our data showed that phosphorylation of MAPKs and STATs proteins is significantly impaired in macrophages from PIP KO mice following stimulation with IFN-γ or LPS. Collectively, these observations indicate that intracellular signaling events leading to phosphorylation of these molecules are impaired in the absence of PIP. It has been previously reported that SOCS1 and SOCS3 are induced following stimulation of macrophages with IFN-γ or LPS and these proteins function to inhibit IFN-γ or LPS-induced signaling events directly by inhibiting the activities of MAPK and Jak kinases (Naka T. *et al.,* 1997; Yasukawa *et al*; 1999). We therefore speculated that deficiency of PIP could enhance the expression of SOCS1 and 3 in PIP KO macrophages leading to the down regulation of phosphorylation of MAPKs and STATs protein. To assess the impact of PIP deficiency on the expression of SOCS 1 and SOCS3, BMDMs from WT and PIP KO mice were stimulated *in vitro* with varying concentrations of LPS and IFN-γ and at different times (0-120 mins), lysed and total cell lysates were assessed for the expression of SOCS1 and SOCS3 by Western blot. Our data showed an increase in the expression of SOCS 1 and SOCS3 protein in BMDMs from PIP KO mice that were significantly higher than in WT macrophages (Fig 17 and 18) (**p <0.01; ***p <0.001).

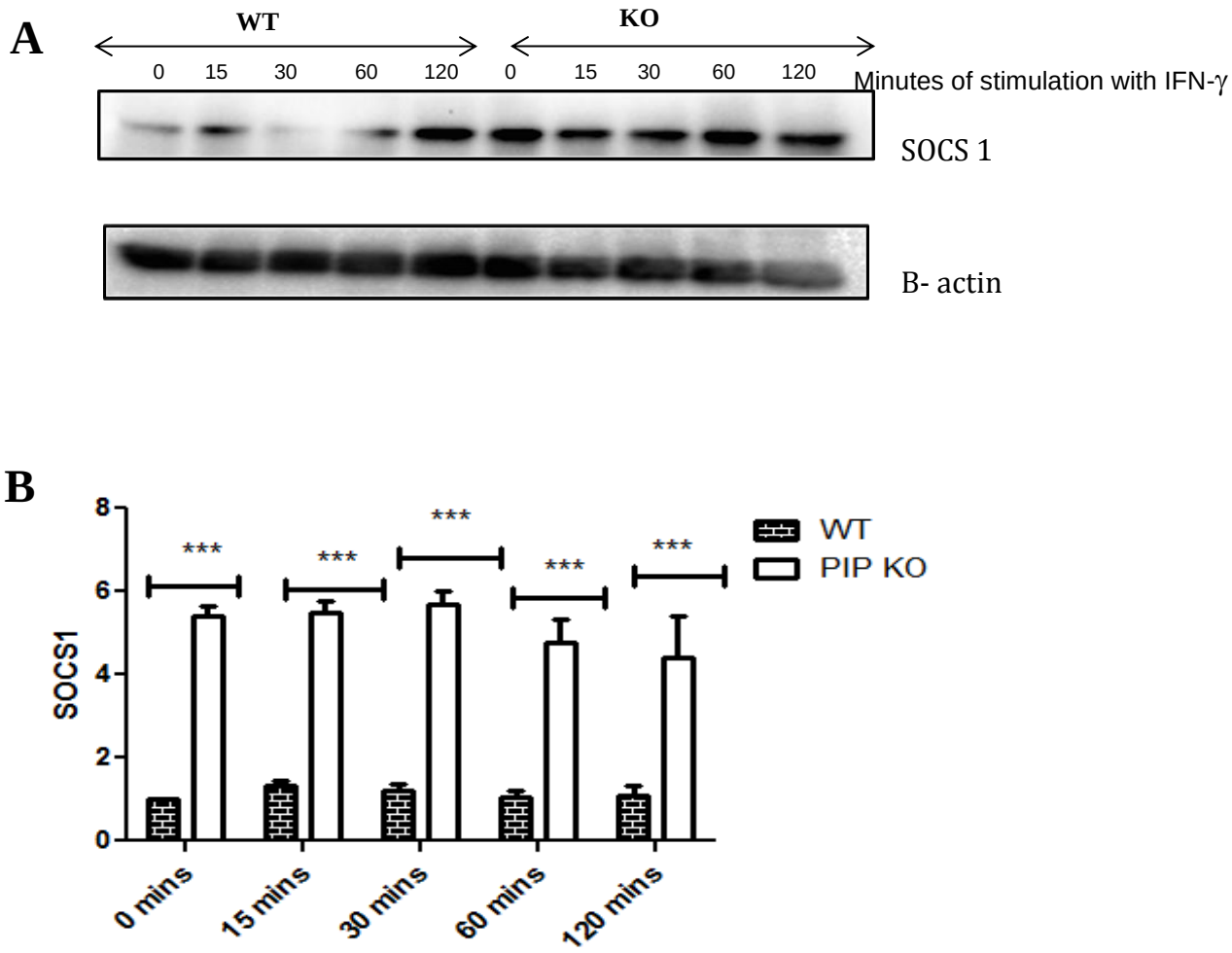


Fig. 17

Increased expression of SOCS1 protein in BMDMs from PIP KO mice stimulated with IFN-γ

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for expression of SOCS1protein by Western blot. **B:** Analysis through densitometry of western blot data probed against SOCS1 and normalized against B- actin levels that represent protein loading; n=3 . (***p <0.001).

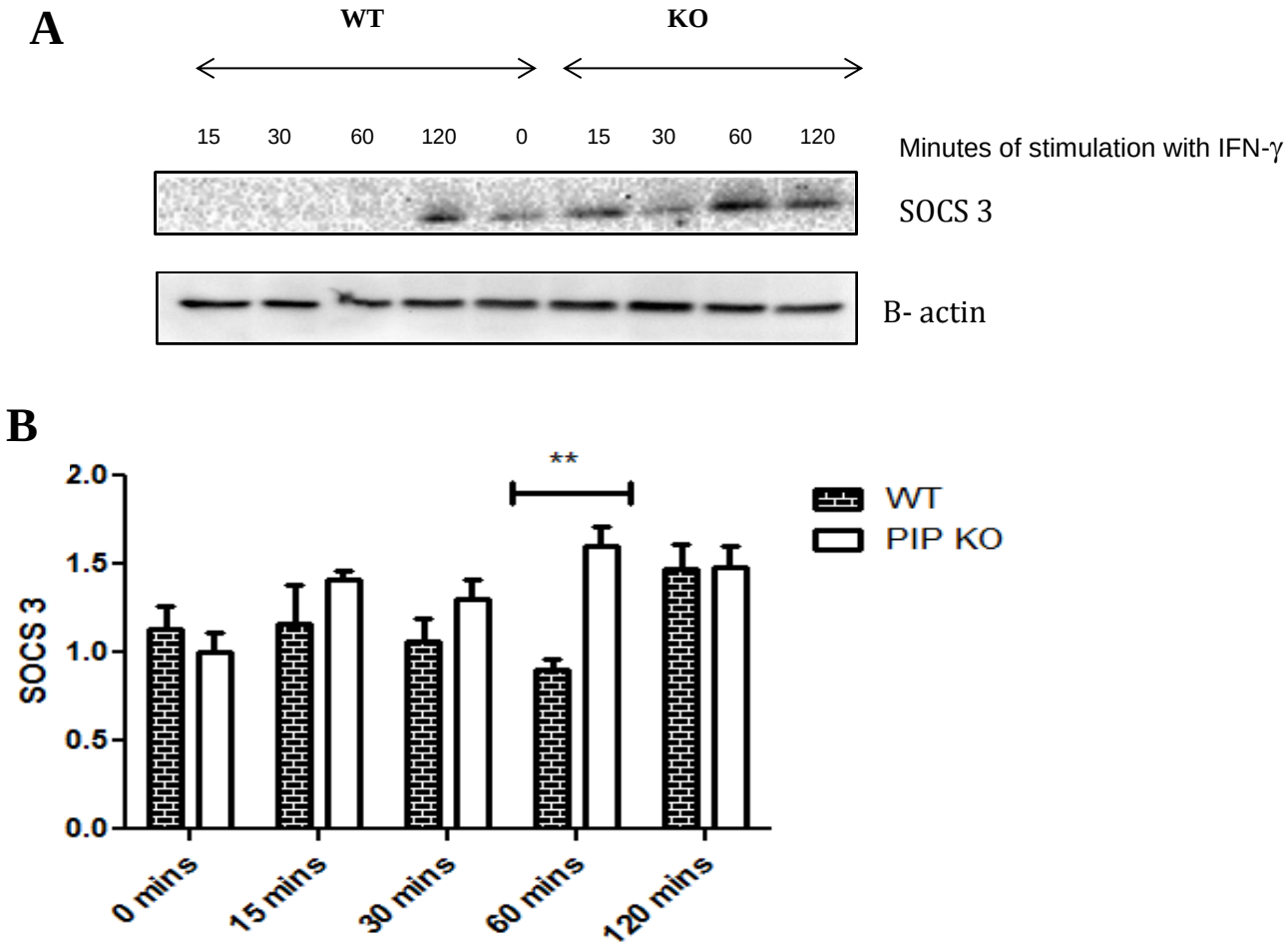


Fig. 18

Increased expression of SOCS 3 protein in BMDMs from PIP KO mice stimulated with IFN-γ

A: BMDMs from WT and PIP KO mice were stimulated *in vitro* with various concentrations of IFN-γ at different times (0-120 mins), lysed and total cell lysates were assessed for expression of SOCS 3protein by Western blot. **B:** Analysis through densitometry of western blot data probed against SOCS 3 and normalized against B- actin levels that represent protein loading; n= 3. (**p <0.01)

6. ASSESSMENT OF PIP EXPRESSION IN BMDMs AND BMDCS FROM WT MICE

Previous work in our lab has demonstrated that prolactin upregulates PIP expression in breast cancer cell lines. More recently, we have shown that macrophages from PIP deficient mice produce significantly less nitric oxide and proinflammatory cytokine than their WT counterparts following LPS or IFN-γ priming. However the expression of PIP in macrophages is yet to be directly demonstrated. To assess this, we cultured and stimulated BMDMs from WT with LPS in the presence or absence of prolactin *in vitro* at different times (0 - 3 hrs) and assessed for the production of PIP by Western blot. As shown in Fig. 19, we were unable to detect PIP protein by western blot, whether the cells were stimulated with prolactin or not (Fig. 19).

Next, we speculated that our inability to detect PIP may be related to translational or posttranslational events that could lead to protein degradation at the time it was made. Therefore, we assessed PIP transcripts (mRNA) in BMDMs, BMDDC, and splenic cells from WT mice following LPS stimulation. As shown in Fig. 20, we still were unable to detect the expression of PIP transcripts (mRNA) from BMDM, BMDDC and splenic cells in WT mice.

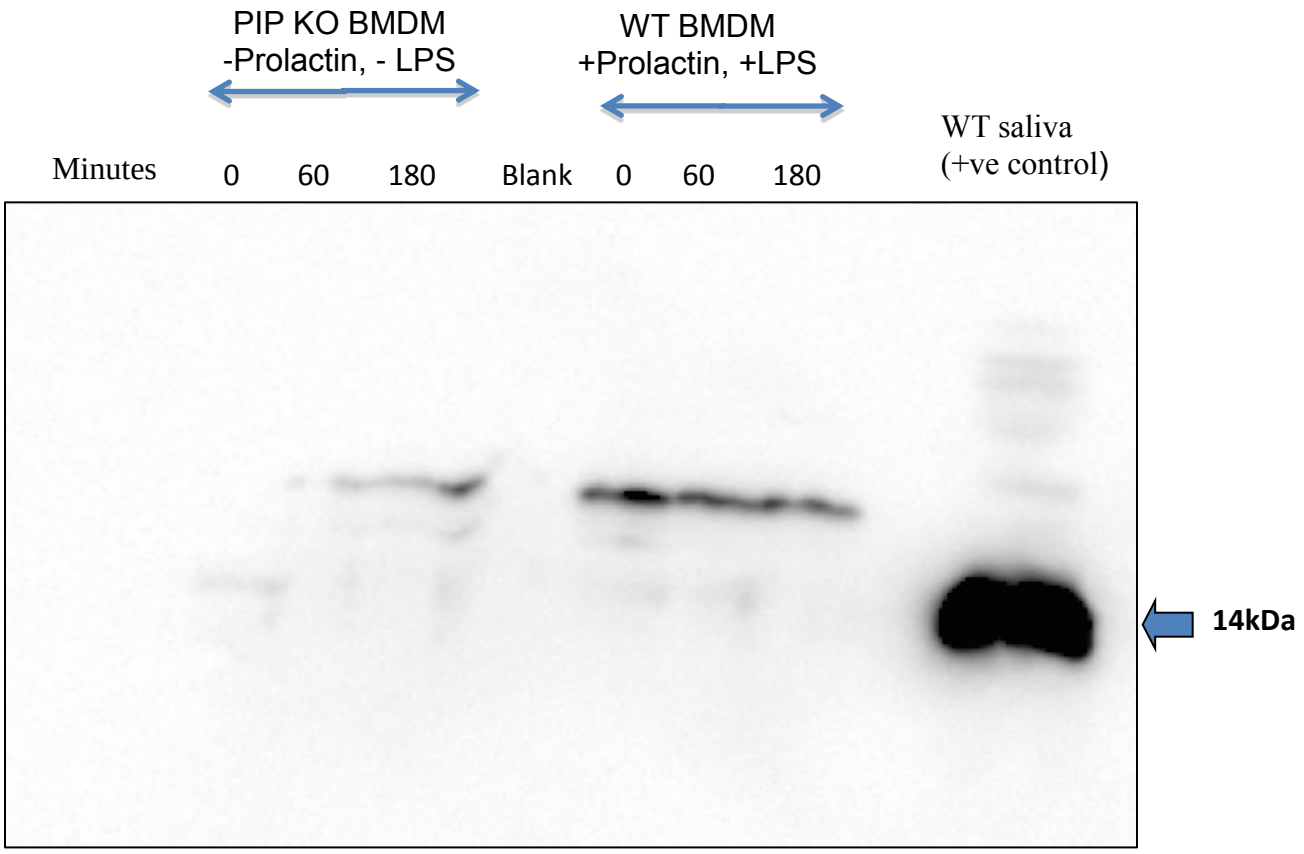
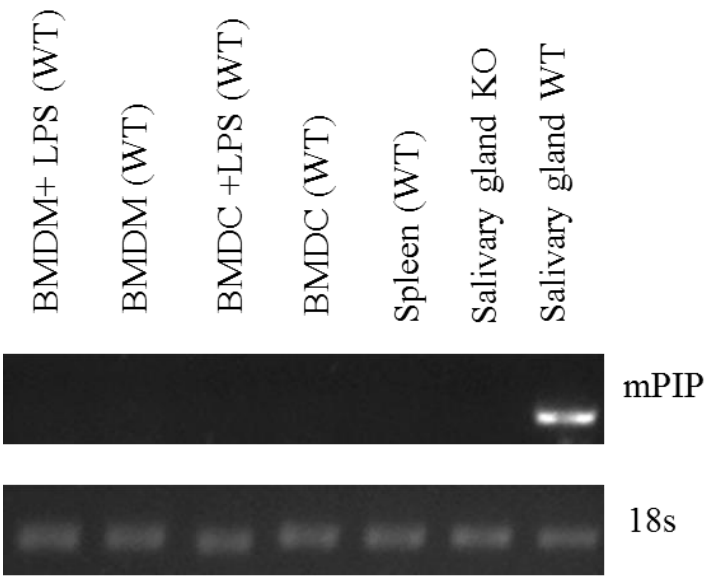


Fig.19

Prolactin does not induce PIP production in BMDM from WT mice:

BMDMs from WT and PIP KO mice were collected. BMDM from WT were cultured and stimulated directly with prolactin and LPS *in vitro* at different times (0 - 3 hrs) while the BMDMs from PIP KO mice serves as a control (Unstimulated with Prolactin). Cells were lysed and total cell lysates were assessed for the production of PIP by Western blot.



Zhirong et al

Fig.20

RT – PCR showing undetected expression of PIP cDNA from BMDM, BMDDC and splenic cells in WT mice:

BMDMs, BMDDC, splenic cells from WT type mice were stimulated with LPS. Both stimulated and unstimulated cells were used and 18S ribosomal RNA serves as a house keeping gene. Cells were lysed and the messenger RNA was converted to cDNA. PIP specific primer was used to amplify cDNA because cDNA contains the message in the cell. We observed impair expression of PIP cDNA from BMDM, BMDDC and splenic cells in WT mice.

SUMMARY OF RESULTS

Data generated from this study shows that:

1. Expression of IFN-γR and TLR4 on macrophage cells derived from bone marrow and splenic cells of WT and PIP KO mice showed no difference.

2. Production of proinflammatory cytokines and NO from bone marrow derived macrophages / dendritic cells of PIP KO mice were impaired when compare to WT counterparts.

3. Deficiency of PIP impairs phosphorylation of MAPKs and STATs protein.

4. Deficiency of PIP upregulates SOCS 1 and SOCS 3 expressions.

5. Direct culturing and stimulation of BMDMs with prolactin does not induce PIP production in WT mice.

6. Failure to detect the expression of PIP transcripts (mRNA) from BMDM, BMDDC and splenic cells in WT mice using RT- PCR

VII. DISCUSSION

The primary aim of this project was to investigate the underlying cause of impaired ability of macrophages from PIP KO mice to produce proinflammatory cytokines and nitric oxide (NO) following LPS and IFN-γ stimulation in order to attempt to determine the differences in resistance of PIP KO and WT mice following *L. major* infection. To this effect, we assessed whether a deficiency of PIP impairs expression of TLRs and IFN-γ receptors on macrophage. Furthermore, we evaluated whether deficiency of PIP impairs intracellular signaling pathways that result in production of NO and pro-inflammatory cytokines.

1. Expression of IFN-γand TLR4 receptors is intact in PIP KO mice

Macrophages are the primary resident cells infected by *Leishmania*, but are also the classical effector cells responsible for killing of parasite. The ability of macrophage to destroy parasite is dependent on their ability to recognize pathogens through their surface receptors resulting in their classical activation by various cytokines signals (IFN-γ and LPS)(Nathan, 1991). It is known that the binding of IFN-γ and LPS to IFN-γR and TLR4 expressed on the surface of macrophages results in their activation and induction of iNOS, which catalyzes the hydrolysis of L-arginine to generate NO, a potent cytotoxic molecule against intracellular parasites, including *Leishmania*. Previous work from our laboratory (*Li et al*., 2015) demonstrated that BMDDCs from PIP KO mice were significantly impaired in NO production following LPS and IFN-γ stimulation and this was associated with a concomitant inability to control parasite proliferation (Li et al., 2015). Whether this impaired generation of NO could have arisen from impaired expression of PIP TLR4 and IFN-γR on macrophages was not tested. We found that BMDMs

and splenic macrophages from WT and PIP KO mice express similar levels of IFN-γR and TLR4 as assessed by flow cytometry. Thus, this observation suggest that the impaired production of NO and proinflammatory cytokines by PIP KO macrophages following IFN-γ or LPS stimulation was not related to impaired expression of these key receptors. Furthermore, this suggest that the inability of BMDMs from PIP KO mice to control *leishmania major* following IFN-γ and LPS stimulation is not associated with impaired expression of IFN-γR and TLR4 on macrophages, but rather could be as a result of impaired signaling activities in the infected cells.

Proinflammatory cytokines are produced by innate immune and non-immune cells following their interaction with pathogen associated molecular patterns (PAMPs) expressed on the surfaces of invading pathogens and play important roles in the initiation and promotion of local and/or systemic inflammatory responses. IL-12 and IL-6 are key proinflammatory cytokines that are associated with the induction of Th1 cell-mediated immune responses against many pathogens in mice including *Leishmania* (Hirano *et al*., 1994). Previous work has shown that the production of IL-12 by *Leishmania*-infected dendritic cells (and to some extent macrophages) and the degree of responsiveness of CD4+ T cells to IL-12 critically affect the outcome of *L. major* infection in mice (vonStebut *et al*., 1998; Berberich *et al*., 2003). Furthermore it has been shown that IL-12 deficient mice on the resistant background are highly susceptible to *L. major* (Sypek *et al*., 1993; Chakir *et al*., 2003). The expression of co- stimulatory molecules and MHC class II are critical for full activation and function of BMDMs and BMDDC to produce proinflammatory cytokines (Banchereau and Steinman, 1998; Inabak *et al*., 2000; Turley *et al*., 2000). Li et al., 2015 showed that BMDDCs from PIP KO mice showed impaired expression of co-stimulatory molecules including CD40, CD80, CD86, and MHC class II (Li *et al*., 2015). However, this impaired

expression of co-stimulatory molecules is unlikely to be the major contributor of impaired Th1 immunity in PIP KO mice because we still observed impaired Th1 differentiation *in vitro* following stimulation of highly purified CD4+ T cells with anti-CD3/anti-CD28 mAb; a condition that bypasses the requirement for co-stimulation *in vivo*.

We found that the production of proinflammatory cytokine by BMDMs and BMDDCs from WT and PIP KO mice were different: following IFN-γ or LPS stimulation, PIP KO cells showed impaired IL-12 and IL-6 production unlike WT cells that showed increased IL-12 and Il-6 production. This impaired production of IL-12 and IL-6 by cells from PIP KO mice was not related to impaired expression of IFN-γ or TLR4 receptors. The finding that BMDMs and BMDDCs from PIP KO significantly produce less IL-12 and IL-6 suggests that PIP might serve as a positive regulator of proinflammatory cytokine production in BMDMs and BMDDC in WT mice. These findings were in agreement with the recent work done by Li et al (Li *et al*., 2015), which showed significantly lower secretion of IL-6, IL-12p40, and TNF from BMDDCs from PIP KO mice following stimulation with LPS or PolyI:C *in vitro.*

The production of proinflammatory cytokines by macrophages and dendritic cells following IFN-γ and LPS stimulation is mediated by signals transmitted through a cascade of intracytoplasmic proteins known as STATs and MAPKs (Wurster *et al.,* 2000). The development of cell-mediated immune responses capable of controlling *Leishmania* infection and resolving disease is critically dependent upon intracellular signaling in macrophages following activation by IFN-γ (Ghosh *et al*., 2008). The production of nitric oxide and proinflammatory cytokines by classically activated macrophages is regulated by intracellular signaling events including phosphorylation of MAPKs and STATs protein

(Vodovotz *et al*., 1993; Yang, et al., 2000). Our previous data showed that LPS and IFN-γ-stimulated BMDMs from PIP KO mice produce significantly less NO than those from WT mice (Li *et al*., 2015). However, the molecular mechanisms that regulate this impairment were not investigated. In this study, we assessed the phosphorylation of different MAPKs and STATs protein from BMDMs of PIP KO and WT mice following stimulation with LPS and IFN-γ. Our result showed dramatic inhibition of phosphorylation of STAT3, ERK, and P38 in PIP KO macrophages stimulated with IFN-γ and LPS. Interestingly, the phosphorylation of STAT1 was not affected, suggesting that there was no global down regulation of phosphorylation in cells from PIP KO mice. The impaired phosphorylation of STAT3 and MAPKs in PIP KO macrophages was associated with a concomitant suppression of NF-κB phosphorylation, a master transcription factor that is critical for the production of proinflammatory cytokines in macrophages. Whether the impaired phosphorylation of MAPKs is directly responsible for the impaired phosphorylation of NF-κB was not investigated in this study. However, it is conceivable that this could directly or indirectly affect NF-κB phosphorylation as it has been shown in other studies. Alternatively, it is plausible that PIP might be regulate NF-κB phosphorylation by enhancing the ability of the inhibitor of Kappa B kinase (IKK) complex to degrade the inhibitor of Kappa B (IkB) proteins, thus promoting the phosphorylation and translocation of NF-κB to the nucleus. Further studies are required to accurately determine the mechanistic pathway through which deficiency of PIP regulates NF-κB phosphorylation in macrophages.

2. PIP: A Negative regulator of SOCS proteins in macrophages

The induction of SOCS1 and SOCS3 proteins in macrophages following LPS or IFN-γ stimulation play a critical role in the direct inhibition of IFN-γ and LPS-induced proinflammatory cytokine responses via the inhibition of Jak kinases activity (Naka T. *et al.*, 1997; Yasukawa *et al*; 1999). We found that BMDMs from PIP KO mice showed impaired phosphorylation of MAPKs and STATs protein following LPS and IFN-γ stimulation. Interestingly, we observed that the expression of SOCS1 protein in BMDMs from PIP KO mice were dramatically increased in unstimulated PIP KO cells (basal expression) and at all-time points following LPS and IFN-γ stimulation compared to those from their WT counterpart mice. Also, the expression of SOCS3 protein was increased precisely at the 60 minute time point in PIP KO mice, although the magnitude was not as high as SOCS1 expression. These observations suggest that PIP might act as a negative regulator of SOCS1 and SOCS3 proteins in macrophages. Thus, in the absence of PIP, unregulated SOCS expression acts to inhibit MAPK and STAT activation/phosphorylation thereby limiting the production of proinflammatory cytokines by macrophages. We do not know whether PIP directly or indirectly associates with SOCS1 and SOCS3, an association that could either affect the stability or degradation of SOCS proteins. Further studies using immunoprecipitation followed by western blotting or CHIP assays could help determine whether there is direct interaction between PIP and SOCS proteins in macrophages.

Despite finding such dramatic phenotype in proinflammatory cytokine production and phosphorylation of MAPKs and STAT3 in macrophages from PIP KO mice following LPS and

IFN-γ stimulation, we were unable to demonstrate PIP expression in these cells either by western blot or RT-PCR. We reasoned that PIP expression in these cells might be upregulated following stimulation with prolactin since the expression of PIP in benign and malignant breast cancer cells and its expression is upregulated by this hormone (Myal *et al*., 1991). Furthermore, prolactin has been shown to be involved in regulating monocyte/macrophage function *in vitro* by binding to its receptor expressed on the surface of macrophages (Aziz *et al*. 2008, Carvalho-Freitas *et al.* 2008). Thus, we incubated macrophages from WT mice with prolactin and LPS, assessed the expression of PIP by western blot. We were still unable to detect PIP expression in these cells. The following factors could contribute to the failure to detect PIP from BMDMs in WT mice:

1. PIP protein could be unstable or degraded during our sample preparation. While this may be plausible, it is highly unlikely because we had previously detected PIP in breast cancer cell lines and salivary glands of WT mice.
2. Failure to titrate the concentration of prolactin used during the experiment to get an optimum concentration. There is no evidence to indicate that the dose of prolactin used in our study is optimal for PIP induction. A carefully titrated dose response curve should be established to determine the optimum dose to be used.
3. Although it has been shown that macrophages expresses prolactin receptors, it is possible that the procedure used in generating these BMDMs might have disrupted the expression of this receptor, which could thus explain the failure to respond to this hormone. It is therefore necessary to assess the expression of this receptor in our BMDMs using flow cytometry.

4. It is possible that while prolactin does not directly influence macrophages *in vitro*, they may do so indirectly *in vivo*. For example, it is conceivable that prolactin acts directly on other cells such as CD4+ T cells, enhancing their release of IFN-γ, which could then indirectly activate macrophages.
5. In this study, we didn't not stimulate with prolactin alone, it could be that LPS is inhibiting the effect of prolactin on the BMDM from WT.
6. It is possible that the stem cells from WT mice do express receptor for PIP such that they have already responded to PIP produced by other epithelial cells at the developmental stage and the effect is imprinted in those stem cell, thus when they are been differentiated into BMDM *in vitro*, they still show an effect unlike in PIP KO mice with impair stem cell PIP receptor and does not show any effect. It is therefore necessary to assess if stem cells do have receptor for PIP.

VIII RELATIONSHIP OF THIS STUDY WITH BREAST CANCER

The nature of an immune response toward an evolving breast tumor is determined by the type of adaptive immunity elicited (DeNardo *et al*., 2007). A tumor-directed immune response involving Th1 cells protects against tumor development and metastasis by secretion on IFN-γ which in turn induces activation macrophage of cytotoxicity (Stout and Bottomly, 1989). Thus, it has been shown that breast cancer patients with large tumors had a more depressed cytokine (IL-12 and IFN-γ) response. This suggests that the infiltration of immune cells (specifically Th1 cells and their cytokines) in a breast cancer tissue that expresses PIP might play a role in limiting progression and metastasis of breast cancer cells. Furthermore, it has been shown that NO

synthase is present in the stroma of breast cancers (Thomsen *et al*., 1995). NO has been suggested to play a dual role in breast cancer and the effects of NO on breast cancer is dependent on the concentration and timing (Jenkins *et al*., 1994; David *et al*., 1998). Thus the presence of a high concentration of NO over a long period of time promotes antitumor activity thereby killing tumor cells and limit metastasis. However at lower concentrations, NO promotes breast tumor growth by supporting angiogenesis (Jenkins *et al*., 1994; David *et al*., 1998). We found that cells from WT mice (which have intact PIP), produce more NO than those from PIP KO mice. Since increased PIP expression is a marker of aggressive cancer and is associated with poor prognostic outcome, it is likely that PIP may play a role in enhancing tumor growth and metastasis by enhancing NO production. In addition, we found that following infection with *L. major*, WT mice showed increased Th1 response and IF-γ production unlike PIP KO mice. Furthermore, we found that CD4+ T cells from PIP KO mice were significantly impaired in their differentiation into IFN-γ-producing Th1 cells following in vitro polarization with anti-CD3/CD28 mAb stimulation. Collectively, these observations suggest that as for proinflammatory cytokines, PIP enhances Th1 cell differentiation and effector cytokine production, an effect that may augment angiogenesis and increased risk of cancer progression.

IX LIMITATIONS OF THIS STUDY

Utilization of an *in vitro* model is the major limitation of this study because some of the results generated *in vitro* might not always correlate with the *vivo* results, thus leading to over – interpretation of the results and erroneous conclusions. In addition, despite the fact that we found impaired phosphorylation of MAPKs and STAT proteins responses in PIP KO mice *in vitro* by

western blotting, it remains to be determined if this impairment can also be observed *in vivo*. This can be done using phospho-flow cytometry to directly analyze surface molecules and intracellular signaling proteins of mice peritoneal macrophages following injection with IFN-γ and LPS.

Another limitation of the current study is the failure to detect PIP *in vitro* both at the protein and mRNA transcript levels in BMDMs derived from WT mice. Although we observed impaired immune response (phosphorylation of MAPKs and STATs protein, production of proinflamatory cytokines and NO) from macrophages from PIP KO mice, we were unable to demonstrate PIP expression in macrophages using different methods (RT-PCR, and Western blot). Thus, we still do not know if macrophages from WT mice produce PIP protein.

X SUMMARY AND CONCLUSION

We have shown that although the expression of IFN-γR and TLR4 are similar in both WT and PIP KO mice, the production of proinflammatory cytokine is different: PIP KO mice showed impaired production of proinflammatory cytokines unlike in WT mice. Also, we have shown that unlike WT mice, BMDMs from PIP KO mice showed impaired phosphorylation of STATs and MAPKs protein involved in IFN-γ signaling. Interestingly, we showed that expression of SOCS1 and SOCS3 proteins, which are known to suppress IFN-γ and LPS signaling, was higher in PIP KO macrophages compared to those from WT mice. This observation stresses the need to evaluate to the mechanism through which SOCS proteins suppress IFN-γ signaling and suggests a potential association and/or crosstalk between SOCS proteins and PIP. Surprisingly, despite the

strong phenotype observed in macrophages from PIP KO mice, we were unable to detect PIP protein or its transcript in primary bone marrow derived macrophages from WT mice.

Collectively, results in this thesis show that although deficiency of PIP does not affect IFN-γR expression on macrophages, it significantly affects intracellular signaling events associated with IFN-γR ligation. This suggests that the inability of PIP KO macrophages to kill *Leishmania* parasites following IFN-γ and LPS stimulation is not due to impaired expression of IFN-γR and TLR4, but may be related in part to impaired intracellular signaling events that are regulated by the concomitant overexpression of SOCS proteins.

XI FUTURE DIRECTIONS

1. Determine how PIP regulates SOCS1 and 3 protein expressions or enhance MAPKs/ STATs phosphorylation

The observation that the expression of SOCS1 and SOCS3 proteins, which are known to suppress IFN-γ and LPS signaling, was higher in PIP KO macrophages compared to those from WT mice, suggests that PIP might act as a negative regulator of SOCS1 and SOCS3 proteins in macrophages and also there could be a direct or indirect association between SOCS proteins and PIP. Thus in WT mice, it could be that PIP binds to SOCS protein and prevent their expression by directly or indirectly affecting the stability of SOCS proteins or enhancing their degradation. To assess if there is a direct interaction between PIP and SOCS protein in BMDMs, immunoprecipitation followed by western blotting or CHIP assays could be used.

2. Phosphorylation of intracellular signaling protein (MyD88) protein from BMDMs in PIP KO and WT mice involved in TLR4 signaling needs to be further investigated.

The response of macrophages to LPS is greatly influenced by MyD88-dependent signaling pathway (Akira *et al*., 2004). The TIRAP–MyD88 dependent pathway plays a key role in regulating early NF-kB activation and pro inflammatory cytokine production such as IL-12. Activation of MyD88 adaptor molecule results in recruitment and activation of the downstream proteins involved in LPS-TLR4 signaling such as TRAF6, IRAK1 and IRAK4. Thus further investigation of MyD88 protein which is the first protein recruited during LPS- TLR4 signaling is necessary. This could be achieved using Western blotting.

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